

1 **The SC-SNc pathway boosts appetitive locomotion in predatory**  
2 **hunting**

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25 **ABSTRACT**

26 Appetitive locomotion is essential for organisms to approach rewards, such as food and  
27 prey. How the brain controls appetitive locomotion is poorly understood. In a naturalistic  
28 goal-directed behavior—predatory hunting, we demonstrate an excitatory brain circuit  
29 from the superior colliculus (SC) to the substantia nigra pars compacta (SNc) to boost  
30 appetitive locomotion. The SC-SNc pathway transmitted locomotion-speed signals to  
31 dopamine neurons and triggered dopamine release in the dorsal striatum. Activation of  
32 this pathway increased the speed and frequency of approach during predatory hunting,  
33 an effect that depended on the activities of SNc dopamine neurons. Conversely, synaptic  
34 inactivation of this pathway impaired appetitive locomotion but not defensive or  
35 exploratory locomotion. Together, these data revealed the SC as an important source to  
36 provide locomotion-related signals to SNc dopamine neurons to boost appetitive  
37 locomotion. (131 words)

## 38 INTRODUCTION

39 Locomotion plays a fundamental role in the survival of organisms. It can be  
40 conceptually divided into three categories: appetitive locomotion, defensive locomotion,  
41 and exploratory locomotion (Sinnamon, 1993). These three types of locomotion may be  
42 selectively recruited by distinct brain circuits for specific behavioral needs (Ferreira-Pinto  
43 et al., 2018). Appetitive locomotion is indispensable for organisms to approach rewarding  
44 targets. For example, in a naturalistic goal-directed behavior—predatory hunting,  
45 predator employs appetitive locomotion to chase and catch up with prey (Hoy et al., 2016;  
46 Han et al., 2017). How the brain control appetitive locomotion during predatory hunting is  
47 an unresolved question in the field of neuroethology (Sillar et al., 2016).

48 The superior colliculus (SC) is a multi-layered midbrain structure for sensory  
49 information processing and motor functions (Cang et al., 2018; Gandhi and Katnani,  
50 2011; Basso and May, 2017). The superficial layers of the SC primarily receive visual  
51 inputs (Morin and Studholme, 2014) and perform visual information processing (Wang et  
52 al., 2010; De Franceschi and Solomon, 2018). The intermediate and deep layers of the  
53 SC are involved in sensorimotor transformation and motor functions (Gandhi and  
54 Katnani 2011). The motor functions of the SC include saccadic eye movement (Wurtz  
55 and Albano, 1980; Sparks, 1986; Wang et al., 2015), head movement (Isa and Sasaki,  
56 2002; Wilson et al., 2018) and locomotion (Cooper et al., 1998; Felsen and Mainen,  
57 2008). From a neuroethological perspective, these motor functions of the SC enable  
58 itself to orchestrate distinct behavioral actions in predatory hunting in rodents (Furigo et  
59 al., 2010; Favaro et al., 2011; Hoy et al., 2019; Shang et al., 2019).

60 How the SC orchestrates distinct behavioral actions during predatory hunting (e.g.  
61 approaching and attacking prey) is beginning to be elucidated. With an unbiased  
62 activity-dependent genetic labeling approach (FosTRAP2), several hunting-associated  
63 tectofugal pathways were identified, such as those projecting to the zona incerta (ZI) and  
64 the substantia nigra pars compacta (SNc) (Shang et al., 2019). While the SC-ZI pathway

65 is primarily involved in sensory-triggered predatory attack during hunting, the functional  
66 role of the SC-SNc pathway in predatory hunting has not been determined yet.

67 The SC-SNc pathway, also known as tectonigral pathway, was first described by  
68 Comoli et al. (2003). It was shown that neurons in the intermediate and deep layers of  
69 the SC form synaptic contacts with dopamine and non-dopamine SNc neurons (Comoli  
70 et al., 2003). Considering the recent studies showing the involvement of SNc dopamine  
71 neurons in the vigor of body movements, including locomotion (Jin and Costa, 2010;  
72 Dodson et al., 2016; Howe and Dombeck, 2016; da Silva et al., 2018; Coddington and  
73 Dudman, 2018), we hypothesized that the SC-SNc pathway may participate in appetitive  
74 locomotion during predatory hunting.

75 In the present study, we explored the role of SC-SNc pathway in appetitive  
76 locomotion during predatory hunting. We found that the SC-SNc pathway transmitted  
77 locomotion speed signals to SNc dopamine neurons and triggered dopamine release in  
78 the dorsal striatum. Activation of this pathway during predatory hunting increased the  
79 speed of appetitive locomotion, an effect that depended on the activities of SNc  
80 dopamine neurons. Conversely, synaptic inactivation of this pathway impaired appetitive  
81 locomotion without changing defensive locomotion. Together, these data revealed the  
82 SC as an important source to provide locomotion-related signals to SNc dopamine  
83 neurons to boost appetitive locomotion.

## 84 RESULTS

### 85 The SC-SNc pathway is primarily glutamatergic

86 We began this study by performing morphological analyses of the SC-SNc pathway.  
87 First, we mapped the SC-SNc pathway with cell-type-specific expression of  
88 "SynaptoTag" (Xu and Südhof, 2013), which is the enhanced green fluorescent protein  
89 fused to synaptic vesicle protein synaptobrevin-2 (EGFP-Syb2). AAV-DIO-EGFP-Syb2  
90 was unilaterally injected into the SC of *vGlut2-IRES-Cre* or *GAD2-IRES-Cre* mice (Figure  
91 1A and 1C). The specificities of *vGlut2-IRES-Cre* and *GAD2-IRES-Cre* mice to label  
92 glutamate+ and GABA+ SC neurons have been validated in an earlier study (Shang et  
93 al., 2019). EGFP-Syb2 expression in SC neurons of *vGlut2-IRES-Cre* mice resulted in  
94 considerable EGFP-Syb2+ puncta in the SNc (Figure 1B and S1A). In contrast, only  
95 sparse EGFP-Syb2+ puncta were observed in the SNc of *GAD2-IRES-Cre* mice (Figure  
96 1D and S1B). We normalized the density of EGFP-Syb2 puncta by dividing the puncta  
97 density in the SNc with that in the SC of each mouse. Strikingly, the normalized density  
98 of EGFP-Syb2 puncta in the SNc of *vGlut2-IRES-Cre* mice was significantly higher than  
99 that of *GAD2-IRES-Cre* mice, suggesting that the SC-SNc pathway is primarily  
100 glutamatergic (Figure 1E).

101 Second, we retrogradely labeled SNc-projecting SC neurons by injecting CTB-555  
102 into the SNc of *WT* mice (Figure 1F). The retrogradely labeled cells (CTB-555+) in the  
103 SC were distributed predominantly in the intermediate and deep layers (Figure 1G and  
104 S1C). By using primary antibodies that specifically recognize GABA and glutamate  
105 (Shang et al., 2018), we found that most of the CTB-555+ cells were  
106 immunohistochemically glutamate+ ( $92\% \pm 3\%$ ,  $n = 5$  mice; Figure 1H) and GABA-  
107 ( $91\% \pm 3\%$ ,  $n = 5$  mice; Figure 1I). These data, again, suggested that the SC-SNc  
108 pathway is primarily glutamatergic.

109 In addition to the SC-SNc pathway, some SC neurons form another pathway to the  
110 ventral tegmental area (VTA) that was implicated in the regulation of sleep and innate  
111 defensive responses (Zhang et al., 2019; Zhou et al., 2019). To test whether  
112 SNc-projecting SC neurons send collaterals to the VTA, we injected CTB-488 and  
113 CTB-555 into the SNc and VTA of the same *WT* mice (Figure S2A). Interestingly, very  
114 few cells were dually labeled by CTB-555 and CTB-488 in the SC (Figure S2, B-E). As a  
115 negative control, injection of mixed CTB-555 and CTB-488 in the SNc (Figure S2F)  
116 resulted in SC neurons co-labeled by both CTB-555 and CTB-488 (Figure S2, G-J).  
117 These data suggested that the SC-SNc pathway is anatomically segregated from the  
118 SC-VTA pathway.

119 We also examined whether the SNc-projecting SC neurons send collaterals to the ZI,  
120 an important center for feeding-related predation (Zhang et al., 2017; Zhao et al., 2019).  
121 CTB-488 and CTB-555 were injected into the SNc and ZI of the same mice (Figure S3A).  
122 Again, very few cells in the SC were co-labeled by CTB-555 and CTB-488 (Figure S3,  
123 B-D), suggesting that the SNc-projecting SC neurons rarely send collaterals to the ZI.

#### 124 **Single SNc-projecting SC neurons encode locomotion speed**

125 To test whether the SC-SNc pathway is involved in locomotion, we made single-unit  
126 recording from the SNc-projecting SC neurons by using antidromic activation strategy  
127 (Shang et al., 2019). AAV-ChR2-mCherry (Boyden et al., 2005) was injected into the SC  
128 of *WT* mice, followed by implantation of an optical fiber above the ChR2-mCherry+ axon  
129 terminals in the SNc (Figure S4A). Three weeks after viral injection, single-unit recording  
130 was performed with a tungsten electrode in the SC of head-fixed awake mice walking on  
131 a cylindrical treadmill (Figure 2A, *left*). The putative SNc-projecting SC neurons were  
132 identified by the antidromic action potentials (APs) evoked by light pulses (473 nm, 1 ms,  
133 2 mW) that illuminated ChR2-mCherry+ axon terminals in the SNc (Figure 2A, *right*). The  
134 antidromically evoked APs had to conform to two criteria (Cohen et al., 2012; Roseberry  
135 et al., 2016): First, their waveform should be similar to that of APs during locomotion;

136 second, their latencies to light pulses should be less than 5 ms. With these empirical  
137 criteria, we identified 18 units as putative SNc-projecting SC neurons. Their  
138 antidromically evoked APs possessed waveforms quantitatively correlated with those of  
139 APs during locomotion (Figure S4B and 2B) and had short response latencies to light  
140 pulses ( $2.7 \text{ ms} \pm 0.4 \text{ ms}$ ,  $n=18$  units; Figure 2C).

141 Then we examined the instantaneous firing rate of these putative SNc-projecting SC  
142 neurons before, during and after locomotion on the treadmill. In general, the activities of  
143 SNc-projecting SC neurons was modulated by locomotion (Movie S1; Figure 2D). To  
144 examine the temporal relationship between the activities of SNc-projecting SC neurons  
145 and locomotion initiation, we aligned firing rate of individual units with the onset of  
146 locomotion (Figure 2E, *top*). We defined the response onset time as the time when the  
147 signal reached 15% of peak amplitude relative to the baseline. The average response  
148 curve started to rise at  $107 \text{ ms} \pm 15 \text{ ms}$  before locomotion onset ( $n = 18$  units; Figure 2E,  
149 *bottom*). Similarly, we aligned firing rate of individual units with the offset of locomotion  
150 (Figure 2F, *top*), and found that the average response curve dropped to baseline at  $121$   
151  $\text{ms} \pm 16 \text{ ms}$  after locomotion offset ( $n = 18$  units; Figure 2F, *bottom*). To examine how the  
152 activities of these units were modulated during locomotion, we plotted the  
153 response-speed curve of each single-unit (Caggiano et al., 2018) and found a correlation  
154 between the firing rate and locomotion speed in the range of  $3 \text{ cm/s} \sim 30 \text{ cm/s}$  (Figure  
155 2G; Spearman correlation coefficient=0.7528;  $P=7.06\text{E-}07$ ). Histological verification  
156 indicated that all the recorded units were localized within the intermediate and deep  
157 layers of the SC (Figure 2H and S4C). These data suggested that the SNc-projecting SC  
158 neurons encode locomotion speed of mice.

### 159 **The SC-SNc pathway promotes appetitive locomotion in predatory hunting**

160 To further explore the role of SC-SNc pathway in locomotion, we examined whether  
161 activation of this pathway promotes locomotion. AAV-ChR2-mCherry was bilaterally  
162 injected into the SC of *WT* mice (Figure 3A and S5A), followed by implantation of an

163 optical fiber above the ChR2-mCherry+ axon terminals in the SNc (Figure 3B). In acute  
164 brain slices with the SC, light pulses (10 Hz, 2 ms, 10 mW) reliably evoked action  
165 potentials from ChR2-mCherry+ SC neurons (Figure S5B). In a linear runway (Figure  
166 3C), photostimulation of the SC-SNc pathway (10 Hz, 20 ms, 6 s, 10 mW) increased  
167 locomotion speed of mice (Figure 3D and 3E). In control mice injected with AAV-mCherry  
168 in the SC, light illumination on mCherry+ axon terminals in the SNc did not promote  
169 locomotion speed (Figure 3D and 3E). In addition, we found that the effects of SC-SNc  
170 pathway activation on locomotion speed depended on the frequency of light stimulation  
171 (Figure S5C).

172 Then we tested whether activation of the SC-SNc pathway boosts appetitive  
173 locomotion during predatory hunting. In predatory hunting, appetitive locomotion  
174 occurred when predator approached prey (Figure 3F). By measuring the instantaneous  
175 azimuth angle and distance between prey and predator (Figure S5D), we were able to  
176 identify a series of intermittent approach episodes (Figure S5E) according to the  
177 established criteria (Hoy et al., 2016). Appetitive locomotion in these approach episodes  
178 was quantitatively assessed by measuring the speed of approach and frequency of  
179 approach. The speed of approach was calculated by averaging the peak speed of each  
180 approach episode in the trial. The frequency of approach was the number of approach  
181 episodes divided by total time of the trial. With the method above, we labeled the  
182 approach episodes (shaded areas in orange) in the behavioral ethogram of predatory  
183 hunting in mice without (OFF) and with (ON) photostimulation of the SC-SNc pathway  
184 (10 Hz, 20 ms, 10 mW) (Movie S2 and S3; Figure 3G and 3H). We found that activation  
185 of the SC-SNc pathway significantly increased the speed of approach (Figure 3I),  
186 increased the frequency of approach (Figure 3J) and reduced the time required for prey  
187 capture (Figure 3K). In contrast, the latency and the frequency of predatory attack with  
188 jaw during hunting were not altered by activation of the SC-SNc pathway (Figure 3, L and

189 M). These data suggested that activation of the SC-SNc pathway specifically promoted  
190 appetitive locomotion during predatory hunting.

### 191 **The SC-SNc pathway is required for appetitive locomotion in predatory hunting**

192 Then we examined whether the SC-SNc pathway is required for appetitive  
193 locomotion during predatory hunting, by synaptically inactivating the SNc-projecting SC  
194 neurons. Tetanus neurotoxin (TeNT), which blocks neurotransmitter release by  
195 proteolytic cleavage of synaptobrevin-2 (Schiavo et al., 1992), has been used as a  
196 molecular tool for synaptic inactivation (Xu and Südhof, 2013; Cregg et al., 2020). The  
197 effectiveness and specificity of TeNT-mediated synaptic inactivation of SC neurons have  
198 been validated in a previous study (Shang et al., 2019). To synaptically inactivate the  
199 SC-SNc pathway, we injected AAV2-retro-mCherry-IRES-Cre (Tervo et al, 2016) and  
200 AAV-DIO-EGFP-2A-TeNT into the SNc and SC of *WT* mice, respectively (Figure 4A).  
201 The injection of AAV2-retro-mCherry-IRES-Cre in the SNc (Figure S6A) and  
202 AAV-DIO-EGFP-2A-TeNT in the SC cooperatively labeled SNc-projecting SC neurons  
203 with EGFP (Figure 4B, *top*), as demonstrated by the co-expression of mCherry and  
204 EGFP in the same SC neurons (Figure 4B, *bottom*).

205 Then we analyzed the effects of SC-SNc pathway inactivation on predatory hunting  
206 behavior in mice. We labeled the approach episodes (shaded areas in orange) in the  
207 behavioral ethogram of predatory hunting in mice without (Ctrl) and with (TeNT) synaptic  
208 inactivation of the SC-SNc pathway (Movie S4 and S5; Figure 4C and 4D).  
209 TeNT-mediated inactivation of SC-SNc pathway impaired predatory hunting by  
210 significantly increasing the time required for prey capture (Figure 4E). Such effect on  
211 hunting could not be explained by an impairment of predatory attack, because neither  
212 the latency nor the frequency of jaw attack during hunting was changed by SC-SNc  
213 pathway inactivation (Figure 4F and 4G). In contrast, both the speed of approach and  
214 frequency of approach in predatory hunting were significantly decreased (Figure 4H and

215 4l). These data suggested that the SC-SNc pathway is required for appetitive locomotion  
216 during predatory hunting.

217 We also tested the role of the SC-SNc pathway in defensive and exploratory  
218 locomotion. In response to looming visual stimuli, mice exhibited escape behavior to  
219 avoid the imminent threats (Yilmaz and Meister, 2013). The escape behavior was used to  
220 measure defensive locomotion (Caggiano et al., 2018). We found that mice without (Ctrl)  
221 and with (TeNT) synaptic inactivation of the SC-SNc pathway exhibited escape followed  
222 by freezing in response to looming visual stimuli (Movie S6 and S7; Figure S6D and  
223 S6E). Quantitative analyses of locomotion speed indicated that synaptic inactivation of  
224 the SC-SNc pathway did not alter the peak speed of escape behavior during looming  
225 visual stimuli (Figure S6F) or average speed after stimuli (Figure S6G), suggesting this  
226 pathway is not required for defensive locomotion. Moreover, these mice exhibited similar  
227 average locomotion speed before looming visual stimuli (Figure S6H), suggesting that  
228 the SC-SNc pathway may not be involved in exploratory locomotion as well.

### 229 **The SC-SNc pathway preferentially innervates SNc dopamine neurons**

230 To explore how the SC-SNc pathway is synaptically connected to the SNc, we  
231 employed mouse lines to genetically label different neuronal subtypes in the SNc. The  
232 most prominent neuronal subtype in the SNc is the dopamine neurons positive for  
233 tyrosine hydroxylase (TH+). These SNc dopamine neurons are largely segregated from  
234 those expressing glutamate decarboxylase-2 (GAD2+) (Tritsch et al., 2014; Kim et al.,  
235 2015) or vesicular glutamate transporter-2 (vGlut2+) (Kawano et al., 2006; Morales and  
236 Root, 2014). Although *TH-GFP* mice (Sawamoto et al., 2001) did not reliably label  
237 dopamine neurons in the VTA, this line marked SNc dopamine neurons with higher  
238 fidelity (Lammel et al., 2015). We confirmed this observation (Figure S7A and S7B) and  
239 used *TH-GFP* mice to genetically label SNc dopamine neurons in this study. By crossing  
240 *Ai14* (Madisen et al., 2010) with *GAD2-IRES-Cre* mice (Taniguchi et al., 2011), we  
241 labeled putative SNc GAD2+ neurons with tdTomato (GAD2-tdT; Figure S7C and S7D).

242 Similarly, *Ai14* was crossed with *vGlut2-IRES-Cre* mice (Vong et al., 2011) to label  
243 putative SNc vGlut2+ neurons with tdTomato (vGlut2-tdT; Figure S7E and S7F).

244 To examine how the SC-SNc pathway synaptically innervates SNc dopamine  
245 neurons and GAD2+ neurons, we generated *GAD2-IRES-Cre/Ai14/TH-GFP* triple  
246 transgenic mice. In this mouse line, putative SNc dopamine neurons were genetically  
247 labeled by GFP (TH-GFP+), whereas putative GAD2+ neurons were identified as those  
248 positive for tdTomato (GAD2-tdT+) (Figure 5A). AAV-ChR2-mCherry was injected into  
249 the SC of *GAD2-IRES-Cre/Ai14/TH-GFP* mice (Figure 5C, *left*). In acute brain slice with  
250 the SNc, we illuminated ChR2-mCherry+ axon terminals with light-pulses (473 nm, 2 ms)  
251 with saturating power (20 mW), while performing whole-cell recordings from TH-GFP+  
252 and adjacent GAD2-tdT+ neurons (Figure 5C, *right*). Using low-chloride internal solution  
253 (Kim et al., 2015), we recorded optogenetically-evoked excitatory postsynaptic currents  
254 (oEPSCs, voltage clamp at -70 mV) and inhibitory postsynaptic currents (oIPSCs,  
255 voltage clamp at 0 mV), which were removed by perfusion of antagonists of glutamate  
256 receptors (APV & CNQX) and GABA<sub>A</sub> receptor (picrotoxin), respectively (Figure S7, G-I).  
257 We found that the amplitude of oEPSCs was significantly higher than that of oIPSCs in  
258 both SNc GAD2-tdT+ and TH-GFP+ neurons (Figure 5, D and E). This was consistent  
259 with the morphological observation that the SNc-projecting SC neurons are primarily  
260 glutamatergic (Figure 1). Moreover, the amplitude of oEPSCs in TH-GFP+ neurons was  
261 significantly higher than that in GAD2-tdT+ neurons (Figure 5, D and E), suggesting that  
262 the SC-SNc pathway preferentially innervate SNc dopamine neurons.

263 To test how the SC-SNc pathway is synaptically connected to SNc vGlut2+ neurons,  
264 we generated *vGlut2-IRES-Cre/Ai14/TH-GFP* triple transgenic mice. In this mouse line,  
265 putative SNc dopamine neurons were genetically labeled by GFP (TH-GFP+), whereas  
266 putative vGlut2+ neurons were identified as those positive for tdTomato (vGlut2-tdT+)  
267 (Figure 5B). We injected AAV-ChR2-mCherry into the SC of triple transgenic mice  
268 (Figure 5C, *left*). In acute brain slices with the SNc, we recorded oEPSCs and oIPSCs

269 from TH-GFP+ and adjacent vGlut2-tdT+ neurons (Figure 5C, *right*). We found that the  
270 amplitude of oEPSCs was significantly higher than that of oIPSCs in both TH-GFP+ and  
271 vGlut2-tdT+ neurons (Figure 5, F and G). Moreover, the amplitude of oEPSCs in  
272 TH-GFP+ neurons was significantly higher than that in vGlut2-tdT+ neurons (Figure 5, F  
273 and G). These data indicated that the SC-SNc pathway has a stronger synaptic  
274 connection with SNc dopamine neurons than their synaptic connections to GAD2+ or  
275 vGlut2+ non-dopamine neurons.

### 276 **Activation of the SC-SNc pathway triggers striatal dopamine release**

277 To further confirm that the SNc dopamine neurons are the postsynaptic target of  
278 SC-SNc pathway, we examined whether activation of this pathway evoke dopamine  
279 release in the dorsal striatum (Lerner et al., 2015). To monitor dopamine release, we  
280 employed genetically encoded GPCR-activation-based dopamine sensor (GRAB<sub>DA</sub>  
281 sensor) that reports dopamine dynamics of nigrostriatal pathway (Sun et al., 2018).  
282 AAV-C1V1-mCherry (Yizhar et al., 2011) and AAV-GRAB<sub>DA</sub> were injected into the SC and  
283 dorsal striatum of *WT* mice (Figure 6A), followed by implantation of optical fibers above  
284 the SNc and dorsal striatum, respectively (Figure 6B). The viral expression and optical  
285 fiber implantation were validated by using immunohistochemistry and slice physiology  
286 (Figure 6, C-F).

287 Then we tested whether activation of the SC-SNc pathway triggers dopamine  
288 release in the dorsal striatum. In freely moving mice, single light-pulses (561nm, 2 ms,  
289 0~20 mW) stimulating the axon terminals of SNc-projecting SC neurons (Figure 6, B and  
290 E) transiently increased the fluorescence of GRAB<sub>DA</sub> sensor in the dorsal striatum  
291 (Figure 6, G and H). As a control, no obvious fluorescence changes were observed in  
292 striatal neurons expressing EGFP (Figure 6, G and H). Moreover, the light-evoked  
293 GRAB<sub>DA</sub> signals were abrogated by D2 receptor antagonist haloperidol (Figure 6, I and  
294 J). These data indicated that SC-SNc pathway activation triggers dopamine release in

295 the dorsal striatum, supporting that the SNc dopamine neurons are the postsynaptic  
296 target of the SC-SNc pathway.

### 297 **SNc dopamine neurons mediate appetitive locomotion evoked by SC-SNc pathway**

298 Then we asked whether the SNc dopamine neurons mediate appetitive locomotion  
299 evoked by SC-SNc pathway. To address this question, we employed the strategy of  
300 designer receptors exclusively activated by designer drugs (DREADD) to  
301 chemogenetically silence SNc dopamine neurons (Armbruster et al., 2007).  
302 AAV-DIO-hM4Di-mCherry and AAV-ChR2-EYFP were injected into the SNc and SC of  
303 *DAT-IRES-Cre* mice (Backman et al., 2006) bilaterally, followed by two optical fibers  
304 implanted above the SNc (Figure 7, A and B). AAV-DIO-mCherry was used as a control  
305 of AAV-DIO-hM4Di-mCherry. In the SC, the expression of ChR2-EYFP and the efficiency  
306 to evoke action potentials from ChR2-EYFP+ neurons were validated (Figure S8A and  
307 S8B). In the ventral midbrain, hM4Di-mCherry was specifically expressed in SNc  
308 dopamine neurons that were intermingled with ChR2-EYFP+ axon terminals from SC  
309 neurons (Figure 7C). Chemogenetic suppression of neuronal firing by Clozapine N-oxide  
310 (CNO, 10  $\mu$ M) was confirmed in slice physiology (Figure 7D).

311 To test whether SNc dopamine neurons mediate the appetitive locomotion evoked by  
312 SC-SNc pathway activation, we intraperitoneally treated the mice with saline or CNO.  
313 Light stimulation of SC-SNc pathway of mice treated with saline significantly increased  
314 the speed of approach (Figure 7E, *left*) and the frequency of approach (Figure 7G, *left*)  
315 during predatory hunting. When the same mice were treated with CNO (1 mg/kg) to  
316 chemogenetically suppress the activities of SNc dopamine neurons, activation of  
317 SC-SNc pathway only mildly increased the speed of approach (Figure 7E, *right*) and the  
318 frequency of approach (Figure 7G, *right*). For each mouse, we calculated “net increase”  
319 of approach speed by subtracting speed of approach during laser OFF from that during  
320 laser ON. It turned out that chemogenetic inactivation of SNc dopamine neurons with  
321 CNO prevented the net increase of approach speed (Figure 7F). Similarly, we calculated

322 “net increase” of approach frequency by subtracting frequency of approach during laser  
323 OFF from that during laser ON. We found that inactivation of SNc dopamine neurons  
324 prevented the net increase of approach frequency during predatory hunting (Figure 7H).  
325 These data suggested that the activities of SNc dopamine neurons are required for the  
326 appetitive locomotion evoked by SC-SNc pathway.

## 327 **DISCUSSION**

328       Appetitive locomotion is required for organisms to approach incentive stimuli in  
329 goal-directed behaviors. How the brain controls appetitive locomotion is poorly  
330 understood. Here we used predatory hunting as a behavior paradigm to address this  
331 question. We demonstrate an excitatory subcortical circuit from the SC to the SNc to  
332 boost appetitive locomotion. The SC-SNc pathway transmits locomotion-speed signals  
333 to dopamine neurons and triggers dopamine release in the dorsal striatum. Activation of  
334 this pathway promoted appetitive locomotion during predatory hunting, whereas synaptic  
335 inactivation of this pathway impairs appetitive locomotion rather than defensive  
336 locomotion. Together, these data reveal the SC as an important source to provide  
337 locomotion-related signals to SNc dopamine neurons to boost appetitive locomotion.

### 338 **The brain circuits for predatory hunting: the SC and beyond**

339       As a naturalistic goal-directed behavior, predatory hunting has been the focus of  
340 studies using diverse animal models, such as toad (Ewert, 1997), zebrafish (Gahtan et  
341 al., 2005; Bianco et al., 2011; Trivedi and Bollmann, 2013) and rodents (Anjum et al.,  
342 2006; Hoy et al., 2016). In these animal models, it was found that the optic tectum (OT)  
343 and its mammalian homolog, the SC, play a fundamental role in predatory hunting (Toad:  
344 Ewert, 1997; Zebrafish: Del Bene et al., 2010; Bianco and Engert, 2015; Rodents: Furigo  
345 et al., 2010; Favaro et al., 2011). In rodents, a recent study has shown that  
346 genetically-defined neuronal subtypes in the SC make distinct contributions to prey  
347 capture behavior in mice (Hoy et al., 2019). The hunting-associated SC neurons may  
348 form divergent neural pathways to orchestrate distinct behavioral actions during  
349 predatory hunting, such as attacking prey (Shang et al., 2019) and, as demonstrated in  
350 this study, appetitive locomotion for approaching prey.

351       In another line of research, it was found that brain areas, which were thought to be  
352 related to food intake, are also involved in predatory hunting. For example, optogenetic

353 activation of GABAergic neurons in the central amygdala (CeA), the lateral  
354 hypothalamus (LH), or the ZI provoked strong predatory hunting in mice (Han et al., 2017;  
355 Li et al., 2018; Zhao et al., 2019). The involvement of feeding-related areas in predatory  
356 hunting may be evolutionarily conserved, because the inferior lobe of hypothalamus in  
357 zebrafish also participates in prey capture behavior (Muto et al., 2017). In addition,  
358 activation of CaMKII $\alpha$ -positive neurons in the medial preoptic area (MPA), which is  
359 related to object craving, also induces hunting-like actions toward prey (Park et al., 2018).  
360 Understanding how the neurons in the SC and in these newly-discovered brain areas  
361 coordinately control predatory hunting is a challenging task for future study.

### 362 **Dopamine system modulates predatory hunting**

363 As an important neuromodulatory system in the brain, dopamine system plays a  
364 critical role in conditioned and unconditioned appetitive behaviors (Schultz, 2007;  
365 Bromberg-Martin et al., 2010). Earlier studies using systemic treatment of agonists or  
366 antagonists of dopamine receptors have demonstrated strong effects of dopaminergic  
367 modulation on predatory hunting in mammals (Schmidt, 1983; Shaikh et al., 1991; Tinsley  
368 et al., 2000). However, two critical questions remained unanswered. First, how is  
369 dopamine system recruited during predatory hunting? Second, considering the multiple  
370 clusters of dopamine neurons in the brain, which specific clusters of dopamine neurons  
371 participate in modulating predatory hunting? In this study, we show that the dopamine  
372 neurons in the SNc are innervated by the SC, a central hub to orchestrate predatory  
373 hunting. The SC-SNc pathway may provide locomotion-related signals to SNc dopamine  
374 neurons to boost appetitive locomotion during predatory hunting. These results may  
375 provide some clues to the above unanswered questions. They supported the recent  
376 studies showing the involvement of SNc dopamine neurons in the vigor of body  
377 movements (Jin and Costa, 2010; Dodson et al., 2016; Howe and Dombeck, 2016; da  
378 Silva et al., 2018; Coddington and Dudman, 2018).

### 379 **More considerations on the functions of SC-SNc pathway**

380 In their seminal studies, Redgrave and colleagues proposed that the SC-SNc  
381 pathway may serve as a route for salient visual stimuli to drive phasic activities of  
382 dopamine neurons (Dommett et al., 2005). In primate, this pathway may mediate  
383 visually-evoked reward expectation signals in dopamine neurons during reinforcement  
384 learning (Takakuwa et al., 2017). In the present study, we recorded single-unit activity of  
385 SNc-projecting SC neurons in head-fixed walking mice (Movie S1), and unexpectedly  
386 found that the SNc-projecting SC neurons encode locomotion speed (Figure 2). This  
387 observation prompted us to examine the role of the SC-SNc pathway in regulating  
388 locomotion during predatory hunting (Figure 3-7). Our data may have added another  
389 perspective for understanding the functions of the SC-SNc pathway. Although we did not  
390 systematically examine the sensory responses of the recorded neurons, we do not rule  
391 out the possibility that these neurons may respond to salient sensory stimuli (e.g. visual  
392 or vibrissal tactile stimuli). In future study, it will be interesting to explore whether the  
393 SC-SNc pathway can integrate both sensory and locomotion-related signals to  
394 dynamically modulate appetitive locomotion during hunting.

### 395 **The origin of locomotion-related signals of SNc-projecting SC neurons**

396 Where do the locomotion-speed signals of the SNc-projecting SC neurons originate?  
397 Several motor-related brain areas (e.g. SNr, PPTg, and motor cortex) directly project to  
398 the SC and may provide motor signals to the SC (Comoli et al., 2012). This speculation  
399 was supported by a recent study showing that the projection from the SNr is the  
400 strongest among the above motor-related brain areas (Doykos et al., 2020). The axons  
401 of GABAergic SNr neurons terminate in the lateral part of deep layers of the SC (Kaneda  
402 et al., 2008), a region that contains SNc-projecting SC neurons studied here. The  
403 inhibition and excitation of SNr neurons well predict the initiation and suppression of  
404 locomotion, respectively (Freeze et al., 2013). These studies suggested that  
405 locomotion-related signals of SNc-projecting SC neurons may at least partially originate  
406 from the SNr, which is the primary output of basal ganglia.

407 **SUPPLEMENTARY INFORMATION**

408 Supplementary information includes eight figures, seven movies and four tables.

409

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416

417 **AUTHOR CONTRIBUTIONS**

418 P.C., C.S., J.Z., M.H., and F.Z. conceived the study. C.S., M.H., Q.P., and A.L. did  
419 injections and fiber implantation. C.S., Z.X., Q.P., H.G., and Y.X. did behavioral tests.  
420 C.S. did single-unit recording. M.H. did histological analyses. C.S. and Z.C. did slice  
421 physiology. Y.W., F.S., Y.L., J.Z., F.Z., M.H., and X.Q. provided reagents. D.L., C.S.,  
422 Z.X., H.G., Z.C. and P.C. analyzed data. P.C. wrote the manuscript.

423

424 **DECLARATION OF INTERESTS**

425 The authors declare no competing financial interests.

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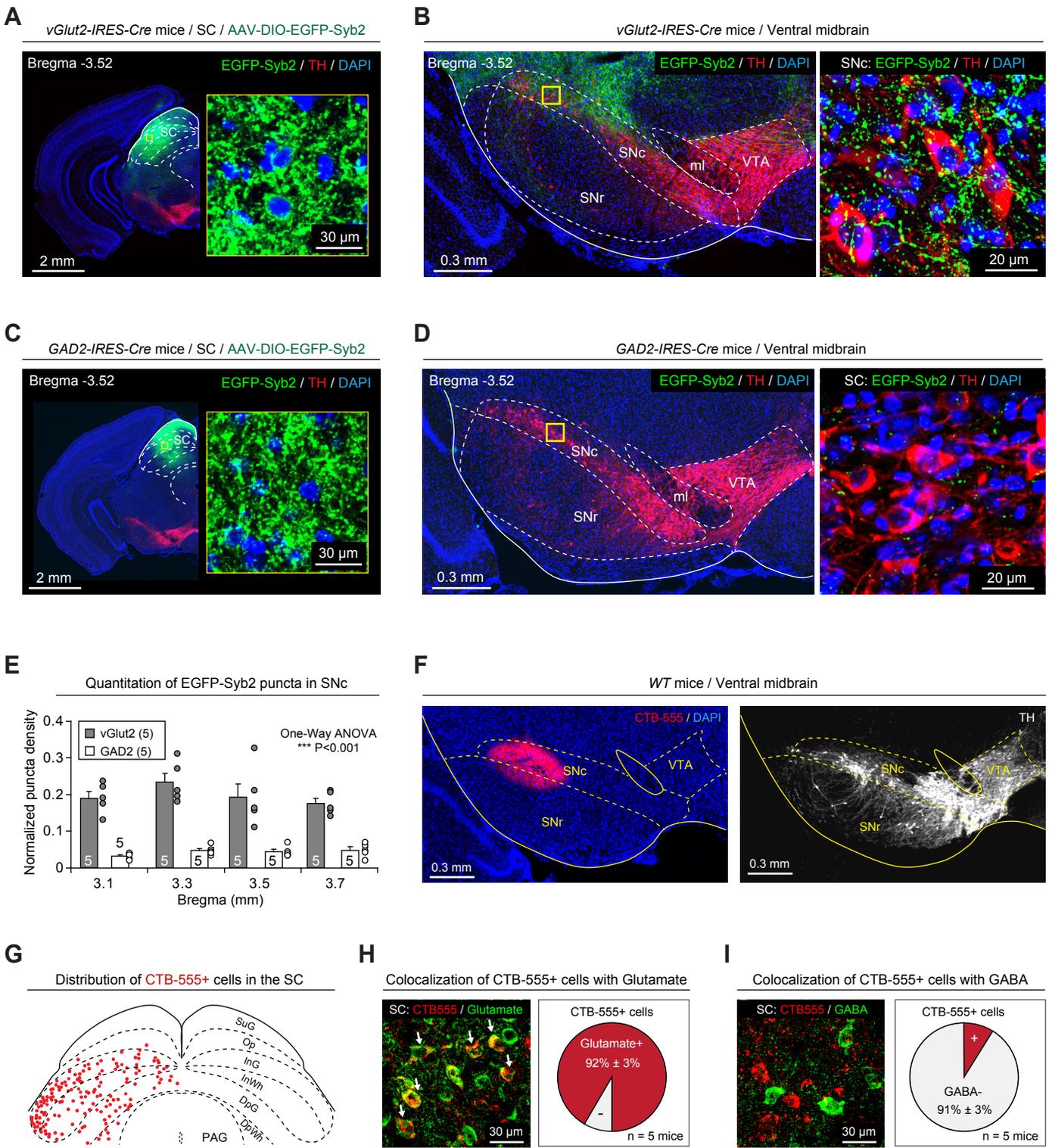


Figure 1 Huang et al., 2020

605 **LEGENDS**

606 **Figure 1 Cell-type-specific mapping of SC-SNc pathway.**

607 **(A, C)** Example coronal brain sections of *vGlut2-IRES-Cre* (A) and *GAD2-IRES-Cre* mice  
608 (C) with EGFP-Syb2 expression in the SC. Insets, high-magnification micrographs  
609 showing EGFP-Syb2+ puncta in the SC. **(B, D)** *Left*, EGFP-Syb2+ axon terminals in the  
610 ventral midbrain of *vGlut2-IRES-Cre* (B) or *GAD2-IRES-Cre* mice (D). *Right*,  
611 high-magnification micrographs showing EGFP-Syb2+ puncta (green) in the SNc. The  
612 boundary of the SNc was delineated according to the immunofluorescence of TH (red).  
613 **(E)** Normalized density of EGFP-Syb2+ puncta in the SNc of *vGlut2-IRES-Cre* (*vGlut2*)  
614 and *GAD2-IRES-Cre* (*GAD2*) mice as a function of bregma. The normalization was  
615 made by dividing the puncta density in the SNc with that in the SC. **(F)** An example  
616 coronal section of ventral midbrain showing the injection of CTB-555 into the SNc of *WT*  
617 mice (*left*). The boundaries of SNc and VTA were determined by immunofluorescence of  
618 TH of dopamine neurons (*right*). **(G)** A schematic diagram showing the distribution of  
619 SNc-projecting SC neurons that were labeled by CTB-555. For the raw image  
620 corresponding to the schematic diagram, see Figure S1C. **(H, I)** Example micrographs  
621 (*left*) and quantitative analyses (*right*) showing CTB-555+ cells in the SC are  
622 predominantly positive for glutamate (H) and negative for GABA (I). Number of mice was  
623 indicated in the graphs (E, H, I). Data in (E, H, I) are means  $\pm$  SEM (error bars).  
624 Statistical analyses in (E) were performed by One-Way ANOVA (\*\*\*)  $P < 0.001$ ). For the P  
625 values, see Table S4. Scale bars are indicated in the graphs.

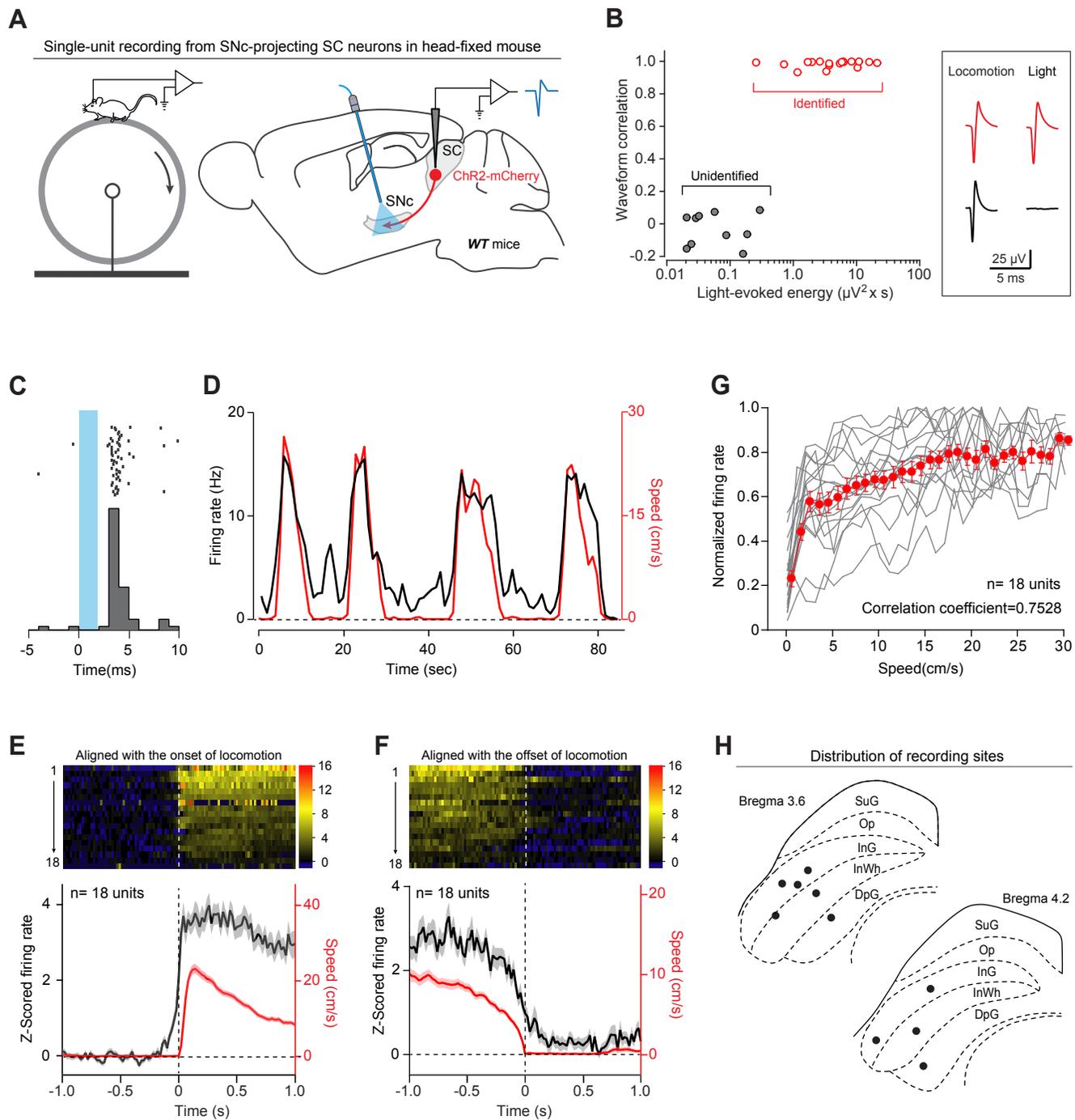


Figure 2 Huang et al., 2020

626 **Figure 2 SNc-projecting SC neurons encode locomotion speed.**

627 **(A)** Schematic diagram showing a head-fixed awake mouse walking on a cylindrical  
628 treadmill (*left*) and antidromic activation strategy for single-unit recording from  
629 SNc-projecting SC neurons (*right*). **(B)** Correlation analysis of action potentials of  
630 individual units evoked either by light pulses (Light) or by locomotion (Locomotion),  
631 confirming a segregation between antidromically identified units (red dots and traces)  
632 and unidentified units (grey dots and traces). **(C)** Raster (*top*) and peristimulus time  
633 histogram (PSTH, *bottom*) of an example putative SNc-projecting SC neurons showing a  
634 latency of ~3 ms relative to the onset of light pulses. **(D)** Smoothed PSTH (trace in black)  
635 of an example putative SNc-projecting SC neuron aligned with locomotion speed (trace  
636 in red). **(E, F)** Heat-map graphs (*top*) and averaged time course (*bottom*) of Z-scored  
637 firing rate of 18 putative SNc-projecting SC neurons aligned to the onset (E) and the  
638 offset (F) of locomotion. **(G)** Averaged (red) and individual (gray) normalized  
639 instantaneous firing rate during locomotion as a function of locomotion speed in 500 ms  
640 bins. **(H)** Distribution of recording site (black dots) in the SC. For example micrograph,  
641 see Figure S4C. Number of units was indicated in the graphs (E-G). Data in (E-G) are  
642 means  $\pm$  SEM (error bars).

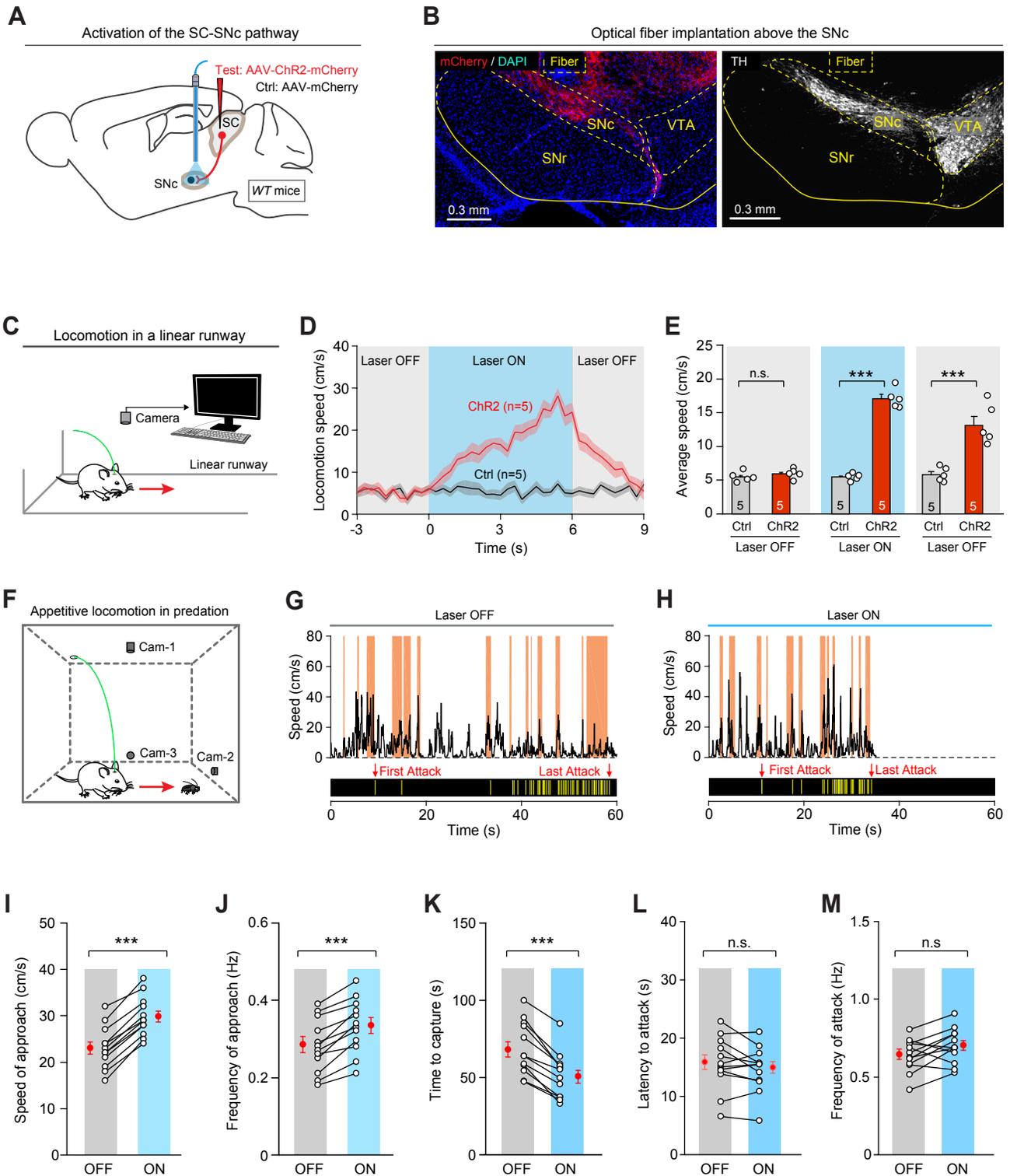


Figure 3 Huang et al., 2020

643 **Figure 3 Activation of the SC-SNc pathway promoted appetitive locomotion. (A)**  
644 Schematic diagram showing injection of AAV-ChR2-mCherry into the SC of *WT* mice,  
645 followed by optical fiber implantation above the SNc. For the example micrograph of  
646 ChR2-mCherry expression in the SC, see Figure S5A. **(B)** An example coronal section of  
647 ventral midbrain with an optical-fiber track above the ChR2-mCherry+ axon terminals in  
648 the SNc (*left*), the boundary of which was delineated by the immunofluorescence of TH  
649 (*right*). **(C)** Schematic diagram showing the experimental configuration to monitor mouse  
650 locomotor behavior in the linear runway. **(D)** Time courses of locomotion speed of control  
651 (Ctrl) and test mice (ChR2) in the linear runway before, during and after light stimulation  
652 of the SC-SNc pathway (10 Hz, 10 ms, 6 s, 10 mW). **(E)** Quantitative analyses of  
653 average locomotion speed of control (Ctrl) and test mice (ChR2) before, during and after  
654 photostimulation of the SC-SNc pathway. For the dependence of locomotion speed on  
655 the frequency of photostimulation, see Figure S5C. **(F)** Schematic diagram showing the  
656 experimental configuration to monitor predatory hunting in the arena. **(G, H)** Time course  
657 of locomotion speed (*top*) and jaw attacks (*bottom*) during predatory hunting of an  
658 example mouse without (G) and with (H) photostimulation of the SC-SNc pathway (10 Hz,  
659 10 ms, 10 mW). The shaded areas (orange) indicated the approach episodes in  
660 predatory hunting. For the analyses of azimuth angle and PPD, see Figure S5F and S5G.  
661 **(I-M)** Speed of approach (I), frequency of approach (J), time to capture (K), latency to  
662 attack (L), and frequency of attack (M) in predatory hunting of mice without (OFF) and  
663 with (ON) photostimulation of the SC-SNc pathway. Number of mice was indicated in the  
664 graphs (D, E, I-M). Data in (D, E, I-M) are means  $\pm$  SEM (error bars). Statistical analyses  
665 in (E, I-M) were performed by Student t-tests (n.s.  $P > 0.1$ ; \*\*\*  $P < 0.001$ ). For the P values,  
666 see Table S4. Scale bars are indicated in the graphs.

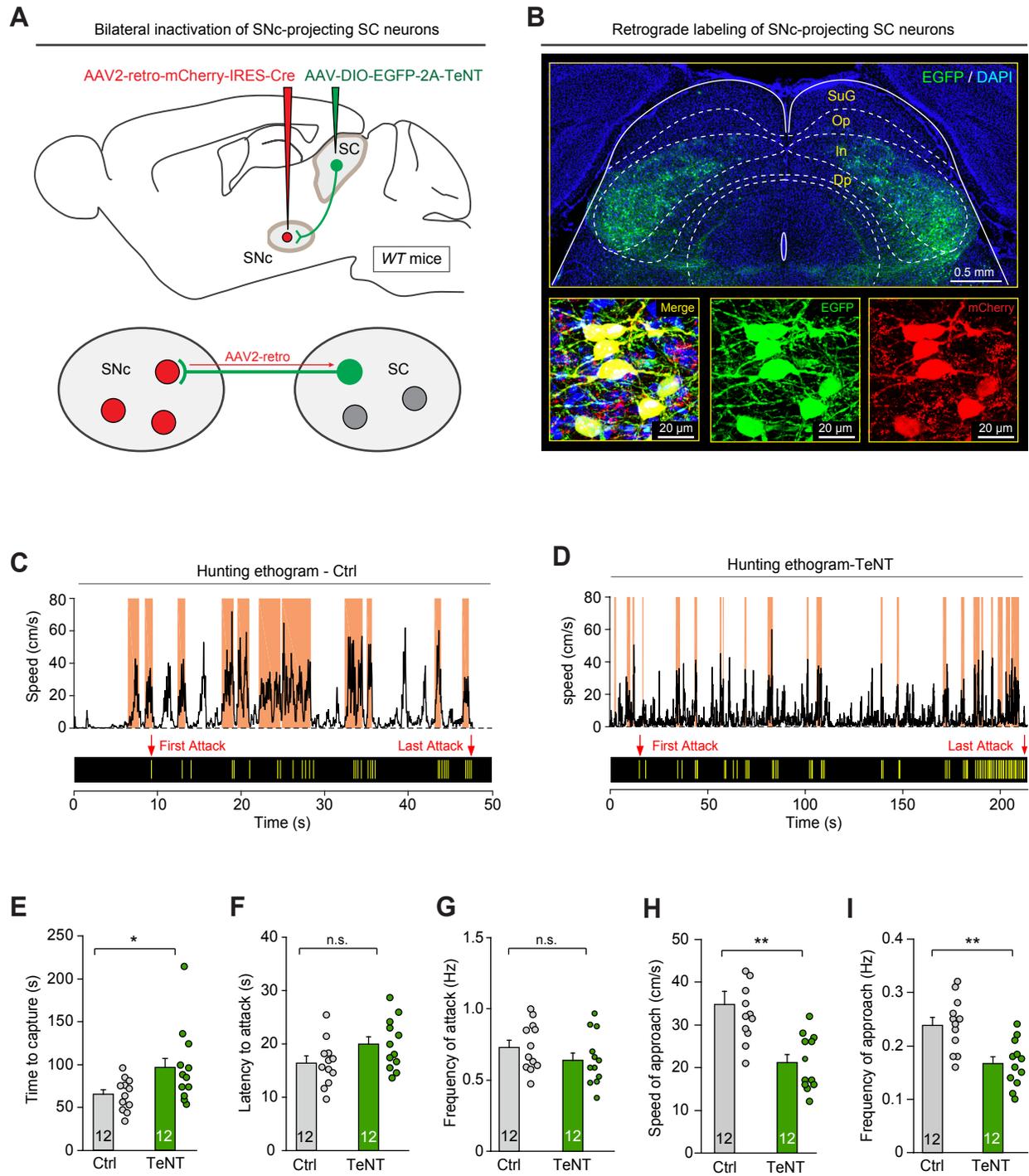


Figure 4 Huang et al., 2020

667 **Figure 4 The SC-SNc pathway is required for appetitive locomotion during**  
668 **predatory hunting.**

669 **(A)** Schematic diagram showing the AAV2-retro strategy to selectively inactivate the  
670 SNc-projecting SC neurons with TeNT. For the example coronal section of ventral  
671 midbrain showing the injection of AAV2-retro-mCherry-IRES-Cre, see Figure S6A. **(B)** An  
672 example coronal brain section showing EGFP<sup>+</sup> SNc-projecting SC neurons distributed in  
673 the intermediate layer (In) and deep layer (Dp) of SC. Inset, merged and single-channel  
674 micrographs showing SNc-projecting SC neurons were dually labeled by mCherry and  
675 EGFP. **(C, D)** Time course of locomotion speed (*top*) and jaw attacks (*bottom*) during  
676 predatory hunting of example mice either without (C, Ctrl) or with (D, TeNT) synaptic  
677 inactivation of SNc-projecting SC neurons. The shaded areas (orange) indicated the  
678 approach episodes in predatory hunting. For the analyses of azimuth angle and PPD,  
679 see Figure S6B and S6C. **(E-I)** Quantitative analyses of time to capture (E), latency to  
680 attack (F), frequency of attack (G), speed of approach (H), and frequency of approach (I)  
681 during predatory hunting in mice without (Ctrl) and with (TeNT) synaptic inactivation of  
682 SNc-projecting SC neurons. Number of mice was indicated in the graphs (E-I). Data in  
683 (E-I) are means  $\pm$  SEM (error bars). Statistical analyses in (E-I) were performed by  
684 Student t-tests (n.s.  $P > 0.1$ ; \*  $P < 0.05$ ; \*\*  $P < 0.01$ ). For the P values, see Table S4. Scale  
685 bars are indicated in the graphs.

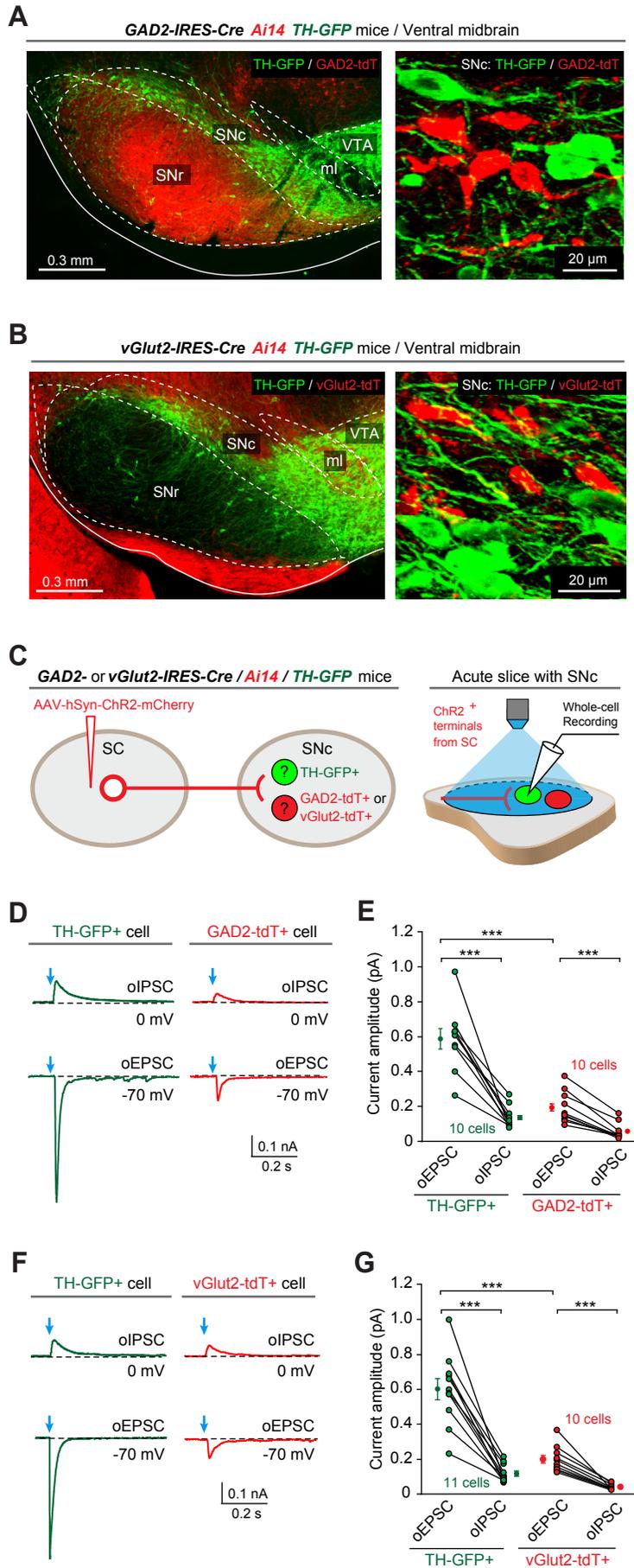


Figure 5 Huang et al., 2020

686 **Figure 5 Dopamine neurons are the primary postsynaptic target of the SC-SNc**  
687 **pathway.**

688 **(A)** An example coronal section with ventral midbrain (*left*) and the high-magnification  
689 micrograph (*right*) showing the segregation of GAD2-tdT+ neurons and TH-GFP+  
690 neurons in the SNc of *GAD2-IRES-Cre/Ai14/TH-GFP* triple transgenic mice. **(B)** An  
691 example coronal section with ventral midbrain (*left*) and the high-magnification  
692 micrograph (*right*) showing the segregation of vGlut2-tdT+ neurons and TH-GFP+  
693 neurons in the SNc of *vGlut2-IRES-Cre/Ai14/TH-GFP* triple transgenic mice. **(C)**  
694 Schematic diagram showing injection of AAV-hSyn-ChR2-mCherry into the SC of  
695 *GAD2-IRES-Cre/Ai14/TH-GFP* or *vGlut2-IRES-Cre/Ai14/TH-GFP* mice (*left*) and  
696 whole-cell recording from TH-GFP+ (green), GAD2-tdT+ (red) or vGlut2-tdT+ (red)  
697 neurons while illuminating ChR2-positive axon terminals from the SC (*right*). **(D, E)**  
698 Example traces (D) and quantitative analyses (E) of oEPSCs and oIPSCs from  
699 TH-GFP+ and GAD2-tdT+ neurons in the slices of *GAD2-IRES-Cre/Ai14/TH-GFP* mice.  
700 **(F, G)** Example traces (F) and quantitative analyses (E) of oEPSCs and oIPSCs from  
701 TH-GFP+ and vGlut2-tdT+ neurons in the slices of *vGlut2-IRES-Cre/Ai14/TH-GFP* mice.  
702 Number of neurons is indicated in the graphs (E, G). Data in (E, G) are means  $\pm$  SEM  
703 (error bars). Statistical analyses in (E, G) were performed by Student t-test (\*\*\*)  $P <$   
704 0.001). For the P values, see Table S4. Scale bars are indicated in the graphs.

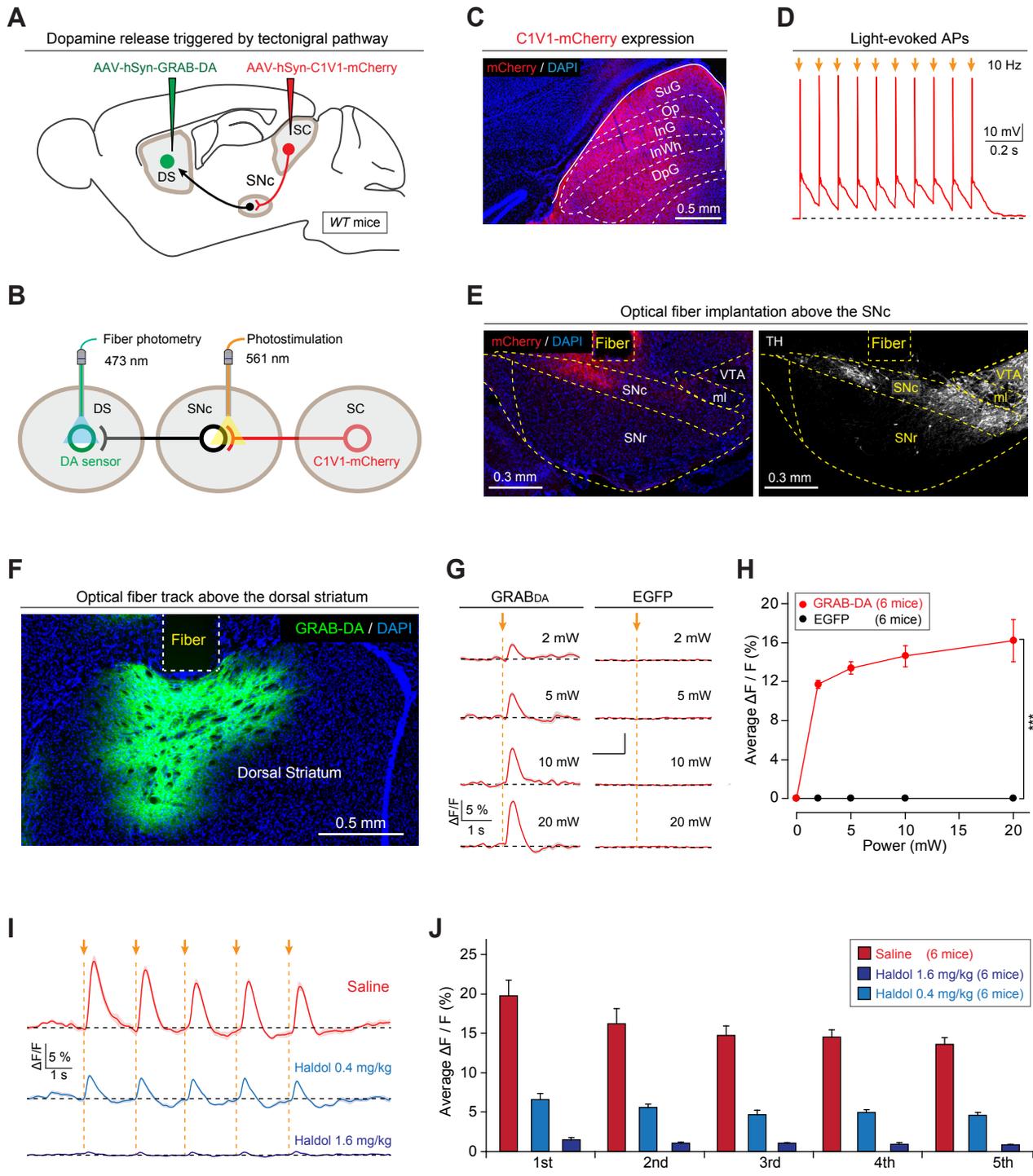


Figure 6 Huang et al., 2020

705 **Figure 6 Activation of the SC-SNc pathway triggers dopamine release in the dorsal**  
706 **striatum.**

707 **(A)** Schematic diagram showing injections of AAV-hSyn-C1V1-mCherry in the SC and  
708 AAV-hSyn-GRAB-DA in the dorsal striatum (DS) of *WT* mice. **(B)** Schematic diagram  
709 showing implantation of optical fibers above the SNc and DS to apply photostimulation  
710 and fiber photometry recording, respectively. **(C)** An example coronal section with  
711 C1V1-mCherry expression in the SC. **(D)** In acute SC slices, light pulses (2 ms, 561 nm,  
712 10 Hz, 10 pulses) illuminating on C1V1-mCherry+ SC neurons reliably triggered action  
713 potential firing. **(E)** An example coronal section of ventral midbrain showing an  
714 optical-fiber track above the C1V1-mCherry+ axon terminals in the SNc (*left*), the  
715 boundary of which was determined according to the immunofluorescence of TH (*right*).  
716 **(F)** An example coronal section with an optical-fiber track above the DS neurons  
717 expressing GRAB-DA sensor. **(G, H)** Example traces (G) and input-output curve (H) of  
718 GRAB-DA signals evoked by photostimulation of the SC-SNc pathway with different  
719 laser power. EGFP was used as a control of GRAB-DA sensor. **(I, J)** Example traces (I)  
720 and quantitative analyses (J) of GRAB-DA signals evoked by photostimulation (561 nm,  
721 5 pulses, 2 ms, 0.5 Hz, 10 mW) of the SC-SNc pathway in mice treated with saline or  
722 Haloperidol (0.4 or 1.6 mg/kg). Number of mice was indicated in the graphs (H, J). Data  
723 in (H, J) are means  $\pm$  SEM (error bars). Statistical analyses in (H, J) were performed by  
724 One-Way ANOVA (\*\*\*)  $P < 0.001$ ). For the  $P$  values, see Table S4. Scale bars are  
725 indicated in the graphs.

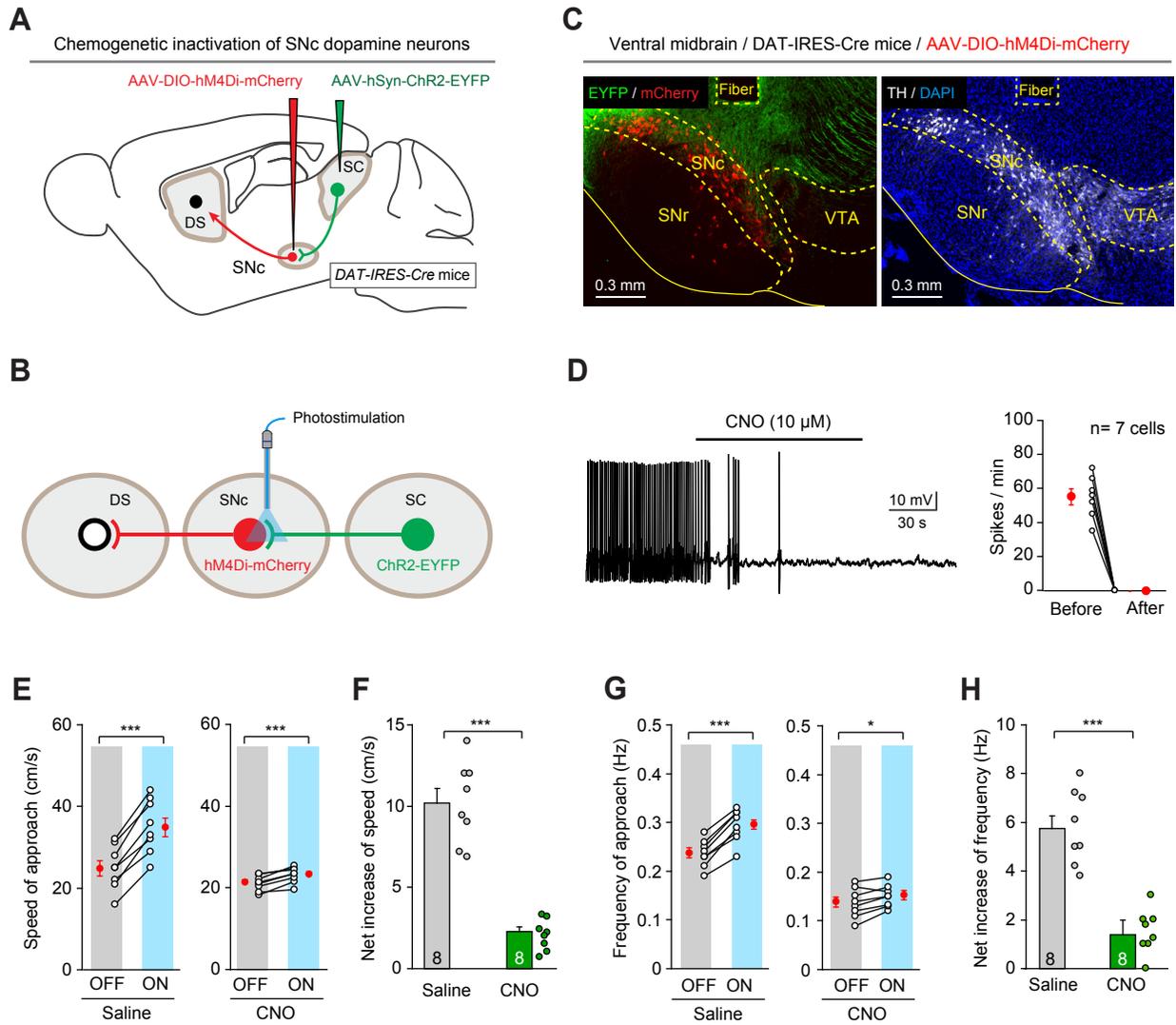


Figure 7 Huang et al., 2020

726 **Figure 7 SNc dopamine neurons mediated the effect of SC-SNc pathway**  
727 **activation.**

728 **(A)** Schematic diagram showing injection of AAV-DIO-hM4Di-mCherry and  
729 AAV-ChR2-EYFP into the SNc and SC of *DAT-IRES-Cre* mice. For an example coronal  
730 section showing the expression of ChR2-EYFP in the SC, see Figure S8A. **(B)**  
731 Schematic diagram showing implantation of an optical fiber above the SNc to apply light  
732 stimulation on ChR2-EYFP+ axon terminals from the SC. **(C)** An example coronal  
733 section of ventral midbrain showing the optical fiber track above the hM4Di-mCherry+  
734 SNc dopamine neurons intermingled with ChR2-EYFP+ axons from the SC (*left*). The  
735 boundaries of SNc and VTA were delineated by the immunofluorescence of TH (*right*). **(D)**  
736 An example train of action potentials recorded from SNc dopamine neurons expressing  
737 hM4Di-mCherry (*left*) and quantitative analyses of spike number per minute before and 3  
738 min after perfusion of 10  $\mu$ M CNO in ACSF (*right*). **(E)** The effect of light stimulation of  
739 the SC-SNc pathway on speed of approach during predatory hunting in mice treated with  
740 saline (*left*) or CNO (*right*). **(F)** Chemogenetic suppression of SNc dopamine neurons  
741 with CNO significantly attenuated the net effects of light stimulation of the SC-SNc  
742 pathway on speed of approach during predatory hunting. **(G)** The effect of light  
743 stimulation of the SC-SNc pathway on frequency of approach during predatory hunting in  
744 mice treated with saline (*left*) or CNO (*right*). **(H)** Chemogenetic suppression of SNc  
745 dopamine neurons with CNO significantly attenuated the net effects of light stimulation of  
746 the SC-SNc pathway on frequency of approach during predatory hunting. Number of  
747 cells (D) and mice (E-H) was indicated in the graphs. Data in (D-H) are means  $\pm$  SEM  
748 (error bars). Statistical analyses in (D-H) were performed by Student t-tests (n.s.  $P > 0.1$ ; \*  
749  $P < 0.05$ ; \*\*\*  $P < 0.001$ ). For the P values, see Table S4. Scale bars are indicated in the  
750 graphs.

## 751 **MATERIALS AND METHODS**

### 752 **Animals**

753 All experimental procedures were conducted following protocols approved by the  
754 Administrative Panel on Laboratory Animal Care at the National Institute of Biological  
755 Sciences, Beijing (NIBS). The *vGlut2-IRES-Cre* (Vong et al., 2011), *GAD2-IRES-Cre*  
756 (Taniguchi et al., 2011), *DAT-IRES-Cre* (Backman et al., 2006), and *Ai14* (Madisen et al.,  
757 2010) mouse lines were imported from the Jackson Laboratory (JAX Mice and Services).  
758 Mice were maintained on a circadian 12-h light/12-h dark cycle with food and water  
759 available ad libitum. Mice were housed in groups (3–5 animals per cage) before they  
760 were separated 3 days prior to virus injection. After virus injection, each mouse was  
761 housed in one cage for 3 weeks before subsequent experiments. To avoid potential  
762 sex-specific differences, we used male mice only.

763

### 764 **AAV vectors**

765 Two AAV serotypes (AAV-DJ, AAV2-retro) were used. The AAVs used in the present  
766 study are listed in Table S1. The viral particles were purchased from Shanghai Taitool  
767 Bioscience Inc. and Brain VTA Inc. The viral vector titers before dilution were in the range  
768 of  $0.8\text{--}1.5 \times 10^{13}$  viral particles/ml. The final titer used for AAV injection is  $5 \times 10^{12}$  viral  
769 particles/ml.

770

### 771 **Stereotaxic injection**

772 Mice were anesthetized with an intraperitoneal injection of tribromoethanol  
773 (125–250 mg/kg). Standard surgery was performed to expose the brain surface above  
774 the superior colliculus (SC), substantia nigra pars compacta (SNc), ventral tegmental  
775 area (VTA), zona incerta (ZI) or dorsal striatum (DS). Coordinates used for SC injection  
776 were as follows: bregma -3.60 mm, lateral  $\pm$  1.30 mm, and dura -1.75 mm. Coordinates

777 used for SNc injection were as follows: bregma -3.40 mm, lateral  $\pm$  1.25 mm, and dura  
778 -4.00 mm. Coordinates used for VTA injection were as follows: bregma -3.40 mm, lateral  
779  $\pm$  0.50 mm, and dura -4.00 mm. Coordinates used for DS injection were as follows:  
780 bregma 0.74 mm, lateral  $\pm$  1.50 mm, and dura -2.40 mm. Coordinates used for ZI  
781 injection were: bregma -2.00 mm, lateral  $\pm$  1.25 mm and dura -4.25 mm. The AAVs and  
782 CTB were stereotaxically injected with a glass pipette connected to a Nano-liter Injector  
783 201 (World Precision Instruments, Inc.) at a slow flow rate of 0.15  $\mu$ l / min to avoid  
784 potential damage to local brain tissue. The pipette was withdrawn at least 20 min after  
785 viral injection.

786 For optogenetic activation and synaptic inactivation experiments, AAV injections  
787 were bilateral. For anterograde and retrograde tracing experiments, CTB injection was  
788 unilateral. Histological analyses were conducted one week (for CTB) and at least three  
789 weeks (for AAV) after injection. Experimental designs related to viral injection are  
790 summarized in Table S2.

791

## 792 **Optical fiber implantation**

793 Thirty minutes after AAV injection, a ceramic ferrule with an optical fiber (230  $\mu$ m in  
794 diameter, numerical aperture = 0.37) was implanted with the fiber tip on top of the SNc  
795 (bregma -3.40 mm, lateral  $\pm$  1.25 mm, dura -3.80 mm) or the dorsal striatum (bregma  
796 0.74 mm, lateral  $\pm$  1.50 mm, and dura -2.20 mm). The ferrule was then secured on the  
797 skull with dental cement. After implantation, the skin was sutured, and antibiotics were  
798 applied to the surgical wound. The optogenetic experiments were conducted 3 weeks  
799 after optical fiber implantation. All experimental designs related to optical fiber  
800 implantation are summarized in Table S2. For optogenetic stimulation, the output of the  
801 laser was measured and adjusted to 2, 5, 10, 15 and 20 mW before each experiment.  
802 The pulse onset, duration, and frequency of light stimulation were controlled by a  
803 programmable pulse generator attached to the laser system.

804

## 805 **Single-unit recording**

806 Antidromic activation strategy was used to identify the single-unit activity of  
807 SNc-projecting SC neurons. AAV-hSyn-ChR2-mCherry was injected into the SC of  
808 wild-type mice, followed by an optical fiber implanted above the SNc. Three weeks after  
809 viral injection, single-unit recording was performed with a tungsten electrode in the SC of  
810 head-fixed awake mouse. The tungsten electrode was vertically advanced into the lateral  
811 SC with a Narishige micro-manipulator. The spikes were amplified by a differential  
812 amplifier (Model 1800, A-M Systems, Everett, WA, USA), digitized (10 kHz) and stored  
813 by Spike2 software (Version 7.03). When the single-unit activity was isolated, we tested  
814 if the units were from SNc-projecting SC neurons. The putative SNc-projecting SC  
815 neurons were identified by the antidromic spikes evoked by light-pulses (473 nm, 1 ms, 2  
816 mW) illuminating ChR2-mCherry+ axon terminals in the SNc. The antidromically evoked  
817 spikes had to conform to two criteria: first, their latency to the light pulse should be less  
818 than 5 ms; second, their waveform should be similar to that of spikes evoked by  
819 locomotion (Figure 2B). Only units with spikes faithfully following the light stimulations  
820 with latency less than 5 ms were further tested for locomotion-correlated activity (Figure  
821 2C). The spike sorting was performed with Spike2 Software (Version 7.03). For a certain  
822 train of action potential, after setting the threshold of the spikes, Spike2 automatically  
823 generated the templates and performed the spike-sorting. The quality of spike clustering  
824 was further confirmed by principal component analysis (Figure S4B). The single-unit  
825 activity of SNc-projecting SC units was recorded with simultaneously measuring the  
826 instantaneous locomotion speed of mice walking on the treadmill (Nanjing Thinktech  
827 Inc.).

828

## 829 **Verification of recording sites**

830 The recording sites of the putative SNc-projecting SC neurons were marked with

831 electrolytic lesions applied by passing positive currents (40  $\mu$ A, 10 s) through the  
832 tungsten electrode. Under deep anesthesia with urethane, the brain was perfused with  
833 saline and PBS containing 4% PFA. After regular histological procedure, frozen sections  
834 were cut at 40  $\mu$ m in thickness and counterstained with DAPI for histological verification  
835 of recording sites.

836

### 837 **Preparation of behavioral tests**

838 After AAV injection and fiber implantation, the mice were housed individually for 3  
839 weeks before the behavioral tests. Before the behavioral tests, they were handled daily  
840 by the experimenters for at least 3 days. On the day of the behavioral test, the mice were  
841 transferred to the testing room and were habituated to the room conditions for 3 h before  
842 the experiments started. The apparatus was cleaned with 20% ethanol to eliminate odor  
843 cues from other mice. All behavioral tests were conducted during the same circadian  
844 period (13:00–19:00). All behaviors were scored by the experimenters, who were blind to  
845 the animal treatments.

846

### 847 **Behavioral paradigm for predatory hunting**

848 The procedure of predatory hunting experiment was described previously (Shang et  
849 al., 2019). Before the predatory hunting test, the mice went through a 9-day habituation  
850 procedure (Day H1–H9). On each of the first three habituation days (Day H1, H2, H3),  
851 three cockroaches were placed in the home-cage (with standard chow) of mice at 2:00  
852 PM. The mice readily consumed the cockroaches within 3 h after cockroach appearance.  
853 On Day H3, H5, H7, and H9, we initiated 24-h food deprivation at 7:00 PM by removing  
854 chow from the home-cage. On Day H4, H6, and H8 at 5:00 PM, we let the mice freely  
855 explore the arena (25 cm x 25 cm) for 10 min, followed by three trials of hunting practice  
856 for the cockroach. After hunting practice, we put the mice back in their home-cages and  
857 returned the chow at 7:00 PM. On the test day, we let the mice freely explore the arena

858 for 10 min, followed by three trials of predatory hunting. After the tests, the mice were put  
859 back in their home-cage, followed by the return of chow. The cockroach was purchased  
860 from a merchant in Tao-Bao Online Stores ([www.taobao.com](http://www.taobao.com)).

861 Before the hunting practice or test, the mice were transferred to the testing room and  
862 habituated to the room conditions for 3 h before the experiments started. The arena was  
863 cleaned with 20% ethanol to eliminate odor cues from other mice. All behaviors were  
864 scored by the experimenters, who were blind to the animal treatments. Hunting  
865 behaviors were measured in an arena (20 cm × 20 cm, square open field) without regular  
866 mouse bedding. After entering, the mice explored the arena for 10 min, followed by the  
867 introduction of a cockroach. For each mouse, predatory hunting was repeated for three  
868 trials. Each trial began with the introduction of prey to the arena. The trial ended when  
869 the predator finished ingesting the captured prey. After the mice finished ingesting the  
870 prey body, debris was removed before the new trial began.

871

### 872 **Measurement of appetitive locomotion and predatory attack in predatory hunting**

873 In the paradigm of predatory hunting, mouse behavior was recorded in the arena with  
874 three orthogonally positioned cameras (50 frames/sec; Point Grey Research, Canada).  
875 With the video taken by the overhead camera, the instantaneous head orientation of  
876 predator relative to prey (azimuth angle) and predator-prey distance (PPD) was analyzed  
877 with the Software EthoVision XT 14 (Noldus Information Technology). The episode of  
878 approach was identified by two empirical criteria (Hoy et al., 2016). First, the PPD should  
879 continuously decrease until it is less than 3 cm. Second, the azimuth angle of mouse  
880 head to cockroach should be within the range of -90 deg to +90 deg. In WT mice, each  
881 trial of predatory hunting contains 10~20 episodes of approach. Speed of approach and  
882 frequency of approach were used to quantitatively measure the appetitive locomotion in  
883 the episodes of approach. Speed of approach of each mouse was calculated by  
884 averaging the peak speed in all the approach episodes in the trial. Frequency of

885 approach was the total number of approach episodes divided by the time to prey capture  
886 in the trial.

887 With the videos taken by the two horizontal cameras, we carefully identified  
888 predatory attacks with jaw by replaying the video frame by frame (50 frames/sec). We  
889 marked the predatory jaw attacks with yellow vertical lines in the behavioral ethogram of  
890 predatory hunting. With this method, we measured three parameters of predatory  
891 hunting: time to prey capture, latency to jaw attack, and frequency of jaw attack. Time to  
892 prey capture was defined as the time between the introduction of prey and the last jaw  
893 attack. Latency to jaw attack was defined as the time between the introduction of the  
894 prey and the first jaw attack from the predator. Frequency of jaw attack was defined as  
895 the number of jaw attacks divided by time to prey capture. Data for three trials were  
896 averaged.

897

#### 898 **Measurement of defensive locomotion triggered by looming visual stimuli**

899 Measurement of defensive locomotion triggered by looming visual stimulus was  
900 described previously (Shang et al., 2018). Briefly, defensive locomotion was measured in  
901 an arena (35 cm × 35 cm, square open field) with corn-cob bedding. No shelter was  
902 provided. A regular computer monitor was positioned above the arena for presentation of  
903 overhead looming visual stimuli. After entering, the mice explored the arena for 10 min.  
904 This was followed by the presentation of three cycles of overhead looming visual stimuli  
905 consisting of an expanding dark disk. The visual angle of the dark disk was expanded  
906 from 2 to 20 degrees within 250 ms. Luminance of the dark disk and background were  
907 0.1 and 3.6 cd/m<sup>2</sup>, respectively. Mouse behavior was recorded (50 frames/sec; Point  
908 Grey Research, Canada) by two orthogonally positioned cameras with LEDs providing  
909 infrared illumination. The instantaneous location of the mouse in the arena was  
910 measured by a custom-written Matlab program. The instantaneous locomotion speed  
911 was calculated with a 200 ms time-bin. The Matlab code is available upon request.

912

### 913 **Measurement of locomotion in linear runway**

914 Mouse behavior was recorded in the linear runway (10 cm x 16 cm x 120 cm) with  
915 an overhead camera (50 frames/sec; Point Grey Research, Canada). With the video  
916 taken by the overhead camera, we measured the instantaneous locomotion speed with  
917 the Software EthoVision XT 14 (Noldus Information Technology).

918

### 919 **Slice physiological recording**

920 Preparation of acute brain slices was performed according to the published work  
921 (Liu et al., 2017). Brain slices containing the SC or SNc were prepared from adult mice  
922 anesthetized with isoflurane before decapitation. Brains were rapidly removed and  
923 placed in ice-cold oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) cutting solution (228 mM sucrose,  
924 11 mM glucose, 26 mM NaHCO<sub>3</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 2.5 mM KCl, 7 mM MgSO<sub>4</sub>, and 0.5  
925 mM CaCl<sub>2</sub>). Coronal brain slices (400 μm) were cut using a vibratome (VT 1200S, Leica  
926 Microsystems, Wetzlar, Germany). The slices were incubated at 28°C in oxygenated  
927 artificial cerebrospinal fluid (ACSF: 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.0  
928 mM MgCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, 15 mM glucose, and 2.0 mM CaCl<sub>2</sub>) for 30 min (~305  
929 mOsm, pH 7.4). The slices were then kept at room temperature under the same  
930 conditions for 30 min before transfer to the recording chamber at room temperature. The  
931 ACSF was perfused at 1 ml/min. The acute brain slices were visualized with a 40×  
932 Olympus water immersion lens, differential interference contrast (DIC) optics (Olympus  
933 Inc., Japan), and a CCD camera.

934 Patch pipettes were pulled from borosilicate glass capillary tubes (Cat #64-0793,  
935 Warner Instruments, Hamden, CT, USA) using a PC-10 pipette puller (Narishige Inc.,  
936 Tokyo, Japan). For recording of postsynaptic currents (voltage clamp), pipettes were  
937 filled with solution (126 mM Cs-methanesulfonate, 10 mM HEPES, 1 mM EGTA, 2 mM  
938 QX-314 chloride, 0.1 mM CaCl<sub>2</sub>, 4 mM Mg-ATP, 0.3 mM Na-GTP, 8 mM

939 Na-Phosphocreatine, pH 7.3 adjusted with CsOH, ~290 mOsm) (Kim et al., 2015). For  
940 recording of action potentials (current clamp), pipettes were filled with solution (135 mM  
941 K-methanesulfonate, 10 mM HEPES, 1 mM EGTA, 1 mM Na-GTP, 4 mM Mg-ATP, pH  
942 7.4). The resistance of pipettes varied between 3.0–3.5 M $\Omega$ . The current and voltage  
943 signals were recorded with MultiClamp 700B and Clampex 10 data acquisition software  
944 (Molecular Devices). After establishment of the whole-cell configuration and equilibration  
945 of the intracellular pipette solution with the cytoplasm, series resistance was  
946 compensated to 10–15 M $\Omega$ . Recordings with series resistances of > 15 M $\Omega$  were  
947 rejected. An optical fiber (230  $\mu$ m in diameter) was used to deliver light pulses, with the  
948 fiber tip positioned 500  $\mu$ m above the brain slices. Laser power was adjusted to 2, 5, 10,  
949 or 20 mW. Light-evoked action potentials from ChR2-mCherry+ neurons in the SC were  
950 triggered by a light-pulse train (473 nm, 2 ms, 10 Hz, 20 mW) synchronized with  
951 Clampex 10 data acquisition software (Molecular Devices). Light-evoked postsynaptic  
952 currents from SNc neurons were triggered by single light pulses (2 ms) in the presence  
953 of 4-aminopyridine (4-AP, 20  $\mu$ M) and tetrodotoxin (TTX, 1  $\mu$ M). D-AP5 (50  $\mu$ M)/CNQX  
954 (20  $\mu$ M) or picrotoxin (PTX, 50  $\mu$ M) were perfused with ACSF to examine the  
955 neurotransmitter/receptor type of optically-evoked postsynaptic currents.

956

### 957 **Fiber photometry**

958 A fiber photometry system (ThinkerTech, Nanjing, China) was used for recording  
959 GRAB<sub>DA</sub> signals from genetically identified neurons (Sun et al., 2018). To induce  
960 fluorescence signals, a laser beam from a laser tube (488 nm) was reflected by a  
961 dichroic mirror, focused by a 10 $\times$  lens (N.A. 0.3) and coupled to an optical commutator. A  
962 2-m optical fiber (230  $\mu$ m in diameter, N.A. 0.37) guided the light between the  
963 commutator and implanted optical fiber. To minimize photo bleaching, the power  
964 intensity at the fiber tip was adjusted to 0.02 mW. The GRAB<sub>DA</sub> fluorescence was  
965 band-pass filtered (MF525-39, Thorlabs) and collected by a photomultiplier tube (R3896,

966 Hamamatsu). An amplifier (C7319, Hamamatsu) was used to convert the photomultiplier  
967 tube current output to voltage signals, which were further filtered through a low-pass filter  
968 (40 Hz cut-off; Brownlee 440). The analogue voltage signals were digitalized at 100 Hz  
969 and recorded by a Power 1401 digitizer and Spike2 software (CED, Cambridge, UK).

970 AAV-DJ-hSyn-GRAB-DA was stereotaxically injected into the dorsal striatum of *WT*  
971 mice followed by optical fiber implantation above the injected site (see “Stereotaxic  
972 injection” and “Optical fiber implantation”). Two weeks after AAV injection, fiber  
973 photometry was used to record GRAB-DA signals from the cell bodies of dorsal striatum  
974 neurons in freely moving mice. A flashing LED triggered by a 1-s square-wave pulse was  
975 simultaneously recorded to synchronize the video and GRAB-DA signals. For recordings  
976 from freely moving mice, mice with optical fibers connected to the fiber photometry  
977 system freely explored the arena for 10 min. After the experiments, the optical fiber tip  
978 sites above the dorsal striatum neurons were histologically examined in each mouse.

979

## 980 **Histological procedures**

981 Mice were anesthetized with isoflurane and sequentially perfused with saline and  
982 phosphate buffered saline (PBS) containing 4% paraformaldehyde (PFA). Brains were  
983 removed and incubated in PBS containing 30% sucrose until they sank to the bottom.  
984 Post-fixation of the brain was avoided to optimize immunohistochemistry of GABA and  
985 glutamate. Cryostat sections (40  $\mu$ m) containing the SC, SNc or DS were collected,  
986 incubated overnight with blocking solution (PBS containing 10% goat serum and 0.7%  
987 Triton X-100), and then treated with primary antibodies diluted with blocking solution for  
988 3–4 h at room temperature. Primary antibodies used for immunohistochemistry are  
989 displayed in Table S1. Primary antibodies were washed three times with washing buffer  
990 (PBS containing 0.7% Triton X-100) before incubation with secondary antibodies (tagged  
991 with Cy2, Cy3, or Cy5; dilution 1:500; Life Technologies Inc., USA) for 1 h at room  
992 temperature. Sections were then washed three times with washing buffer, stained with

993 DAPI, and washed with PBS, transferred onto Super Frost slides, and mounted under  
994 glass coverslips with mounting media.

995 Sections were imaged with an Olympus (Japan) VS120 epifluorescence microscope  
996 (10× objective lens) or an Olympus FV1200 laser scanning confocal microscope (20×  
997 and 60× oil-immersion objective lens). Samples were excited by 488, 543, or 633 nm  
998 lasers in sequential acquisition mode to avoid signal leakage. Saturation was avoided by  
999 monitoring pixel intensity with Hi-Lo mode. Confocal images were analyzed with ImageJ  
1000 software.

1001

### 1002 **Quantification of synaptic puncta density**

1003 The micrographs used for measuring puncta density of SynaptoTag (Figure 1, B and  
1004 D) were acquired with a 63× objective of Zeiss confocal system and analyzed with NIH  
1005 Image J. The analysis of the synaptic puncta was described previously (Cao et al., 2013).  
1006 In brief, the scale of micrographs was set in NIH Image J based on the physical  
1007 dimension of micrographs acquired by Zeiss confocal system. After converting the  
1008 micrographs from RGB color mode to 16-bit mode, the puncta in micrographs were  
1009 binarized and the puncta density was measured automatically by NIH Image J. Then the  
1010 puncta density in the SNc of each mouse was normalized by dividing with that in the  
1011 intermediate layer of the lateral SC (Figure 1E).

1012

### 1013 **Cell-counting strategies**

1014 Cell-counting strategies are summarized in Table S3. For counting cells in the SC,  
1015 we collected 40- $\mu$ m coronal sections from bregma -3.28 to bregma -4.48 for each mouse.  
1016 Six sections evenly spaced by 200  $\mu$ m were sampled for immunohistochemistry to label  
1017 cells positive for different markers. We acquired micrographs (10× objective, Olympus  
1018 FV1200 microscope, Japan) within intermediate and deep layers of the SC followed by  
1019 cell counting with ImageJ software. We calculated the percentages of glutamate+ and

1020 GABA+ neurons in the neuronal population retrogradely labeled by CTB-555. For  
1021 counting cells in the SNc, we collected coronal sections (40  $\mu$ m) from bregma -2.80 to  
1022 bregma -3.80 for each mouse. Five sections evenly spaced by 200  $\mu$ m were sampled for  
1023 immunohistochemistry to label SNc cells positive for different markers. After image  
1024 acquisition, we outlined the SC and SNc followed by cell counting with ImageJ software.  
1025 The boundary of SNc in coronal sections was identified based on TH staining.

1026

## 1027 **DATA QUANTIFICATION AND STATISTICAL ANALYSIS**

1028 All experiments were performed with anonymized samples in which the  
1029 experimenter was unaware of the experimental conditions of the mice. For the statistical  
1030 analyses of experimental data, Student t-test and One-Way ANOVA were used. The “n”  
1031 used for these analyses represents number of mice or cells. See the detailed information  
1032 of statistical analyses in figure legend and in Table S4.

1033

## 1034 **DATA AND CODE AVAILABILITY**

1035 The data that support the findings of this study are available from the corresponding  
1036 author upon reasonable request. The MATLAB code for data analyses is available from  
1037 the corresponding author upon request.

1038

1039 77. Liu Z, et al. IGF1-Dependent Synaptic Plasticity of Mitral Cells in Olfactory Memory  
1040 during Social Learning. *Neuron* 95, 106-122 e105 (2017).

1041 78. Shang C, et al. Divergent midbrain circuits orchestrate escape and freezing  
1042 responses to looming stimuli in mice. *Nature Communications* 9, 1232 (2018).

1043 79. Cao P, et al., Complexin activates exocytosis of distinct secretory vesicles controlled  
1044 by different synaptotagmins. *Journal of Neuroscience* 33 (4), 1714-1727 (2013)

## **Supplementary Materials**

### **The SC-SNc pathway boosts appetitive locomotion in predatory hunting**

Meizhu Huang, Dapeng Li, Qing Pei, Zhiyong Xie, Huating Gu, Aixue Liu, Zijun Chen, Yi Wang, Fangmiao Sun, Yulong Li, Jiayi Zhang, Miao He, Yuan Xie, Fan Zhang, Xiangbing Qi, Congping Shang, Peng Cao

This file includes:

Figure S1 to Figure S8

Movies S1 to S7

Tables S1 to S4

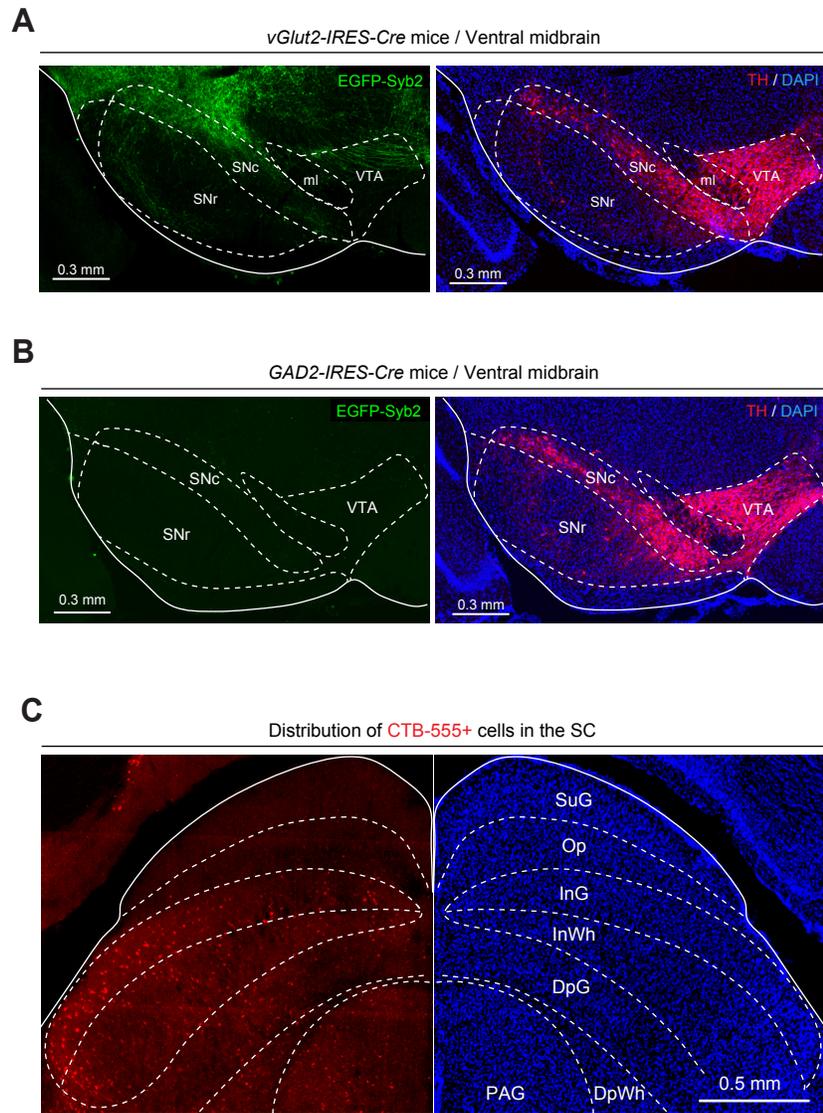


Figure S1 Huang et al., 2020

1 **Figure S1 Cell-type-specific mapping of tectonigral pathway.** (Related to Figure 1)

2 **(A, B)** Single-channel and merged micrographs showing EGFP-Syb2+ axon terminals in  
3 the ventral midbrain of *vGlut2-IRES-Cre* (A) and *GAD2-IRES-Cre* mice (B). The  
4 boundaries of SNc and VTA were determined by immunostaining of tyrosine hydroxylase  
5 (TH, red) for dopamine neurons. **(C)** An example coronal section of the SC showing the  
6 distribution of SNc-projecting SC neurons that were labeled by CTB-555. Scale bars  
7 were indicated in the graphs.

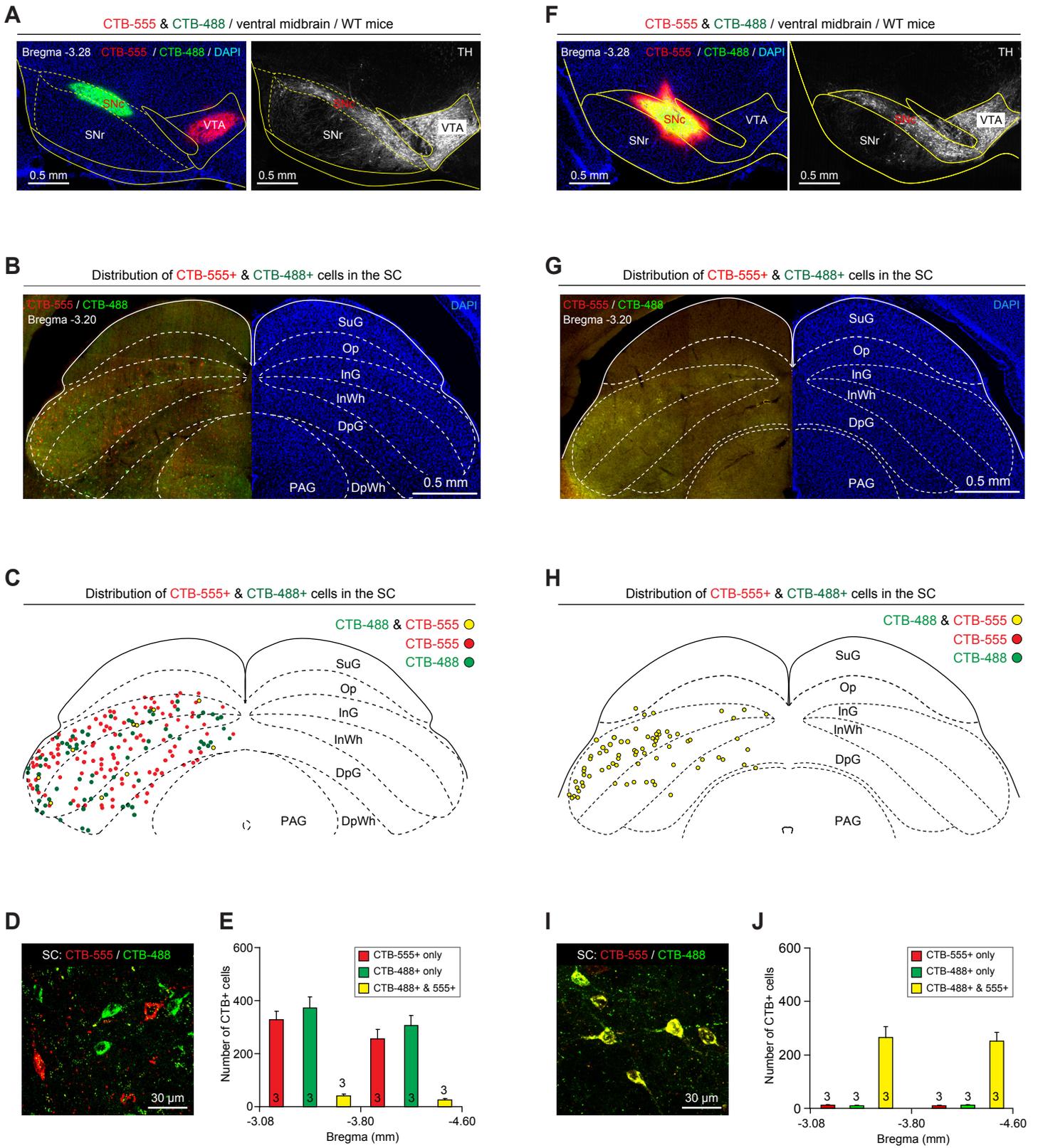


Figure S2 Huang et al., 2020

8 **Figure S2 SC-SNc pathway and SC-VTA pathway are anatomically segregated.**

9 (Related to Figure 1)

10 **(A)** Example coronal section of the ventral midbrain showing injection of CTB-488 and  
11 CTB-555 into the SNc and VTA (*left*), the boundaries of which were delineated according  
12 to the immunofluorescence of TH (*right*). **(B, C)** An example coronal section of the SC (B)  
13 and the corresponding illustration (C) showing the distribution of CTB-555+ & CTB-488+  
14 cells in the SC. **(D, E)** Example micrograph (D) and quantitative analyses (E) showing  
15 CTB-555+ & CTB-488+ SC neurons were largely segregated. **(F)** Example coronal  
16 section of the ventral midbrain showing injection of mixed CTB-488 & CTB-555 into the  
17 SNc (*left*), the boundary of which was delineated according to the immunofluorescence  
18 of TH (*right*). **(G, H)** An example coronal section of the SC (G) and the corresponding  
19 illustration (H) showing the distribution of CTB-555+ & CTB-488+ cells in the SC. **(I, J)**  
20 Example micrograph (I) and quantitative analyses (J) showing the retrogradely labeled  
21 SC neurons are mostly positive for both CTB-555 and CTB-488. Numbers of mice (E and  
22 J) are indicated in the graphs. Data in (E and J) are means  $\pm$  SEM. Scale bars are  
23 labeled in the graphs.

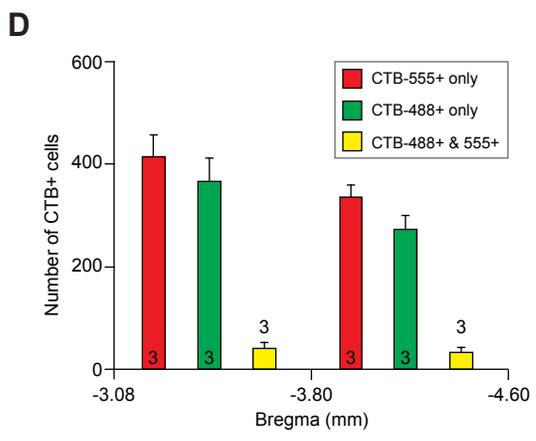
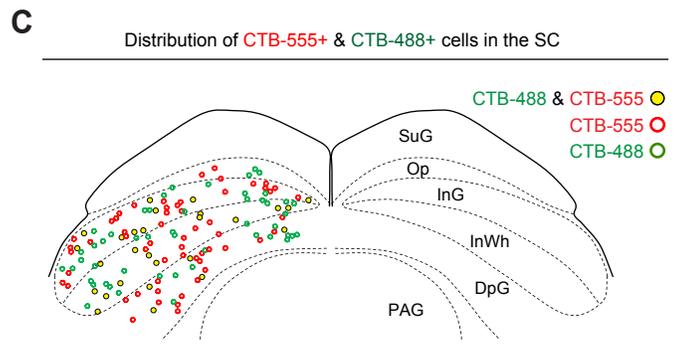
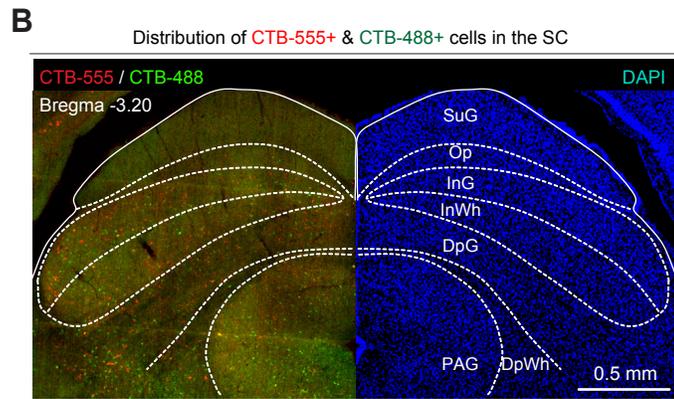
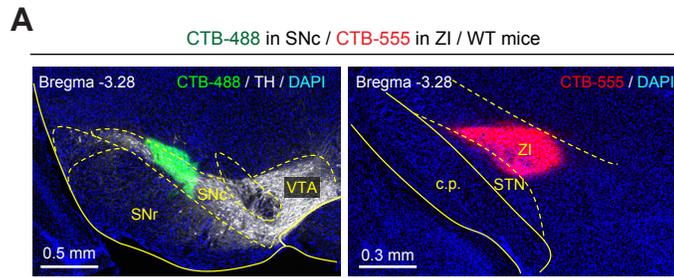


Figure S3 Huang et al., 2020

24 **Figure S3 SC-SNc pathway and SC-ZI pathway are anatomically segregated.**

25 (Related to Figure 1)

26 **(A)** Example coronal brain section showing injection of CTB-488 and CTB-555 into the  
27 SNc (*left*) and ZI (*right*), respectively. The boundary of SNc was delineated according to  
28 the immunofluorescence of TH (*left*). **(B, C)** An example coronal brain section (B) and the  
29 corresponding illustration (C) showing the distribution of CTB-555+ & CTB-488+ cells in  
30 the SC. **(D)** Quantitative analyses showing CTB-555+ & CTB-488+ cells in the SC were  
31 largely segregated. Numbers of mice (D) are indicated in the graphs. Data in (D) are  
32 means  $\pm$  SEM. Scale bars are labeled in the graphs.

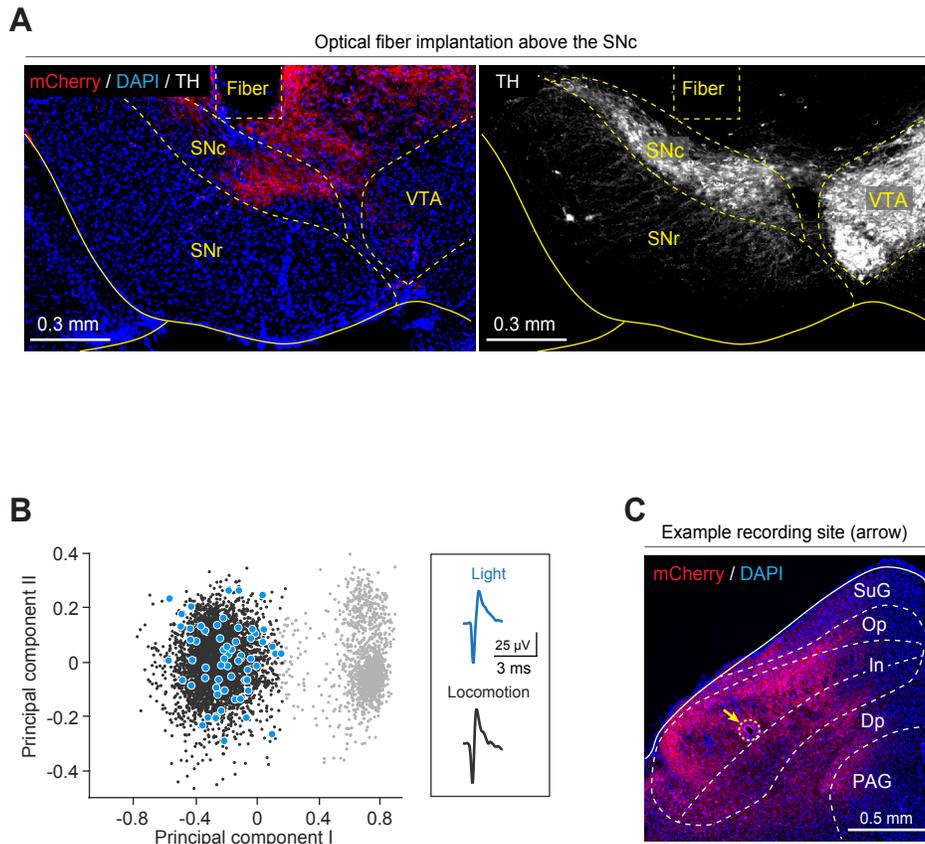


Figure S4 Huang et al., 2020

33 **Figure S4 SNc-projecting SC neurons encode the speed of locomotion.** (Related to  
34 Figure 2)

35 **(A)** Example coronal section of the ventral midbrain showing the optical-fiber track above  
36 ChR2-mCherry<sup>+</sup> axon terminals in the SNc (*left*), the boundary of which was determined  
37 by immunofluorescence of TH for dopamine neurons (*right*). **(B)** Principal component  
38 analyses of light-evoked spikes (blue) and locomotion-evoked spikes (black) of an  
39 example putative SNc-projecting SC neuron. Gray dots, noise. **(C)** Example coronal  
40 section of the SC showing a recording site marked by electrolytic lesion (arrow) in the  
41 intermediate layer (In) of the SC. Scale bars are labeled in the graphs.

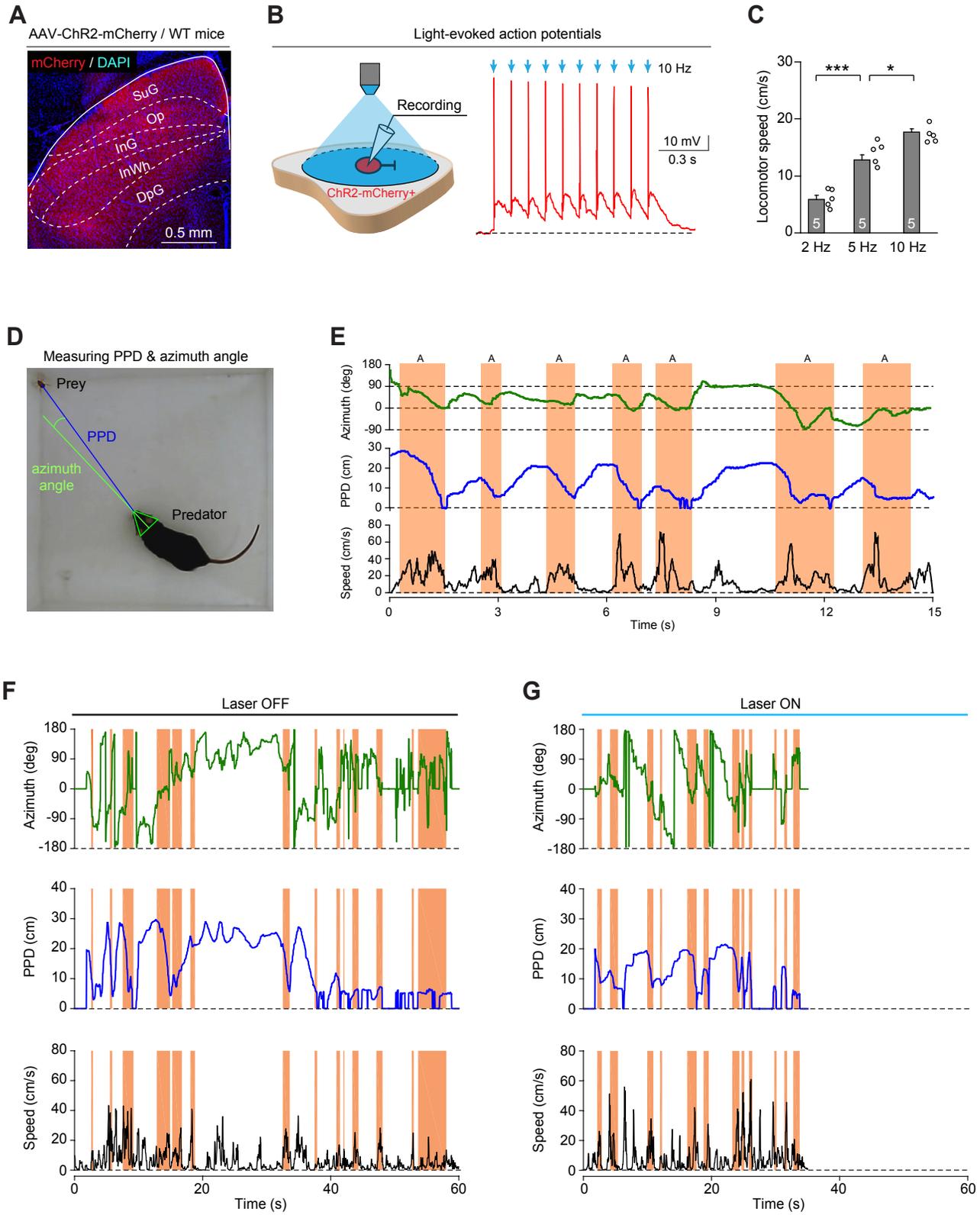


Figure S5 Huang et al., 2020

42 **Figure S5 Activation of the SC-SNc pathway promotes appetitive locomotion**  
43 **during predatory hunting.** (Related to Figure 3)

44 **(A)** Example coronal section of the SC showing ChR2-mCherry expression in the SC of  
45 WT mice. **(B)** Schematic diagram (*left*) and example trace (*right*) showing light pulses (2  
46 ms, 473 nm, 10 Hz, 10 pulses) reliably triggered action potential firing from  
47 ChR2-mCherry<sup>+</sup> SC neurons in acute SC slices. **(C)** Quantitative analyses of average  
48 locomotion speed of mice in the linear runway during photostimulation of the SC-SNc  
49 pathway (10 ms, 6 s, 10 mW) with different frequencies (2 Hz, 5 Hz, 10 Hz). **(D)** An  
50 example picture showing computer-aided measurement of azimuth angle and  
51 prey-predator distance (PPD) when predator approached prey. **(E)** Aligned time courses  
52 of azimuth angle (*top*), prey-predator distance (PPD, *middle*), and locomotion speed  
53 (*bottom*) of an example mouse in predatory hunting, showing the identification of  
54 approach episodes (shaded areas in orange). The intermittent approach episodes were  
55 characterized by azimuth within a narrow range (-90 – 90 deg), by decreased PPD and  
56 by pulses of locomotion speed. For the detailed criteria to identify the approach episodes,  
57 see Methods. **(F, G)** Time courses of azimuth angle (*top*), PPD (*middle*), and locomotion  
58 speed (*bottom*) during predatory hunting of an example mouse without (F, Laser OFF)  
59 and with (G, Laser ON) photostimulation of the SC-SNc pathway. Numbers of mice (C)  
60 are indicated in the graphs. Data in (C) are means  $\pm$  SEM. Statistic analyses (C) were  
61 performed using Student t-test (\*\*\*)  $P < 0.001$ ; \*  $P < 0.05$ ). For the P values, see Table S4.  
62 Scale bars are labeled in the graphs.

**A**

Ventral midbrain / AAV2-retro-mCherry-IRES-Cre / WT mice

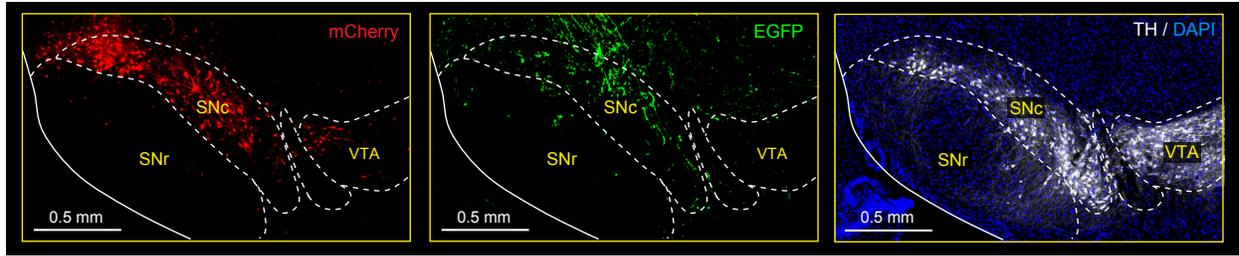
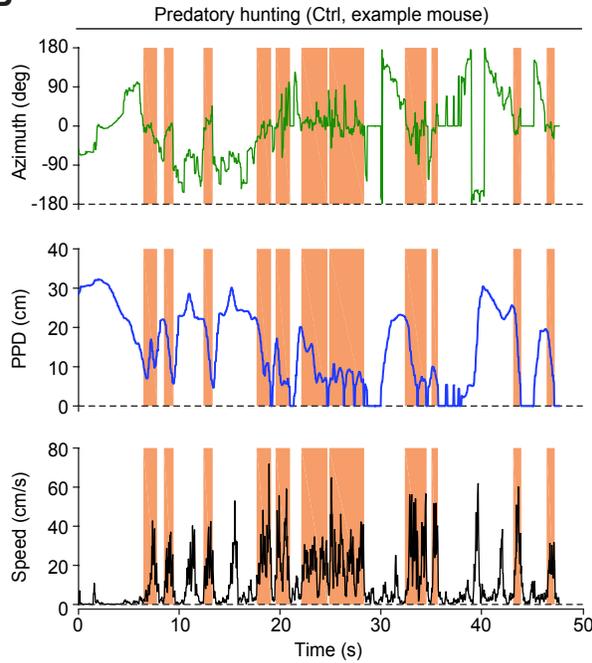
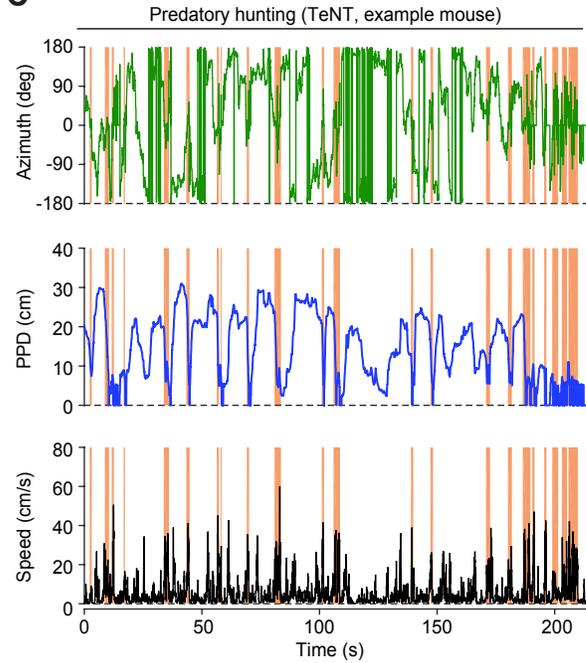
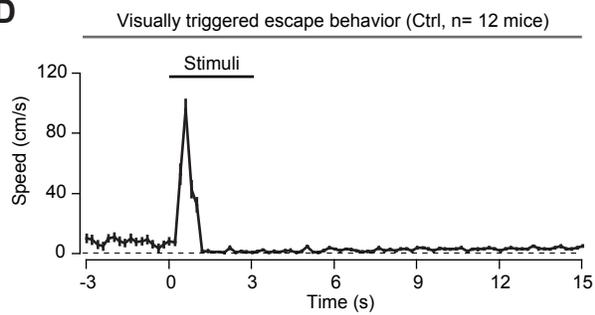
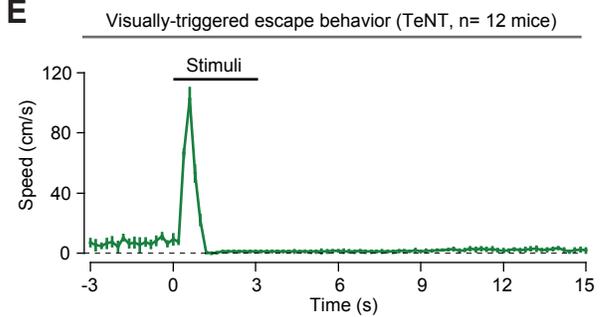
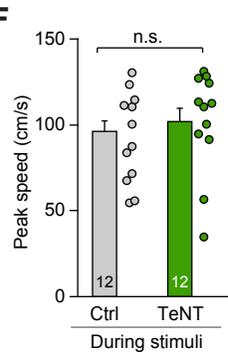
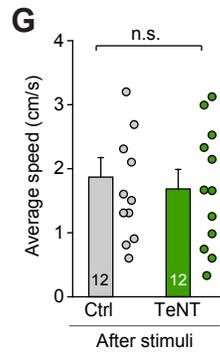
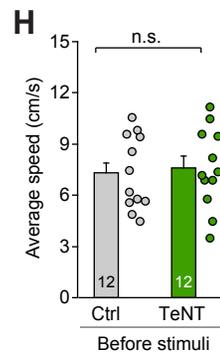
**B****C****D****E****F****G****H**

Figure S6 Huang et al., 2020

63 **Figure S6 The SNc-projecting SC neurons are required for appetitive locomotion**  
64 **during predatory hunting.** (Related to Figure 4)

65 **(A)** Example coronal section of ventral midbrain showing mCherry+ cells in the SNc (*left*)  
66 are intermingled with EGFP+ axon terminals from SC neurons (*middle*). The boundary of  
67 the SNc is determined by immunofluorescence of TH (*right*). **(B, C)** Time courses of  
68 azimuth angle (*top*), PPD (*middle*), and locomotion speed (*bottom*) during predatory  
69 hunting of example mice without (B, Ctrl) and with (C, TeNT) synaptic inactivation of the  
70 SNc-projecting SC neurons. **(D, E)** Time courses of locomotion speed before, during and  
71 after looming visual stimuli in mice without (Ctrl, D) and with (TeNT, E) synaptic  
72 inactivation of SNc-projecting SC neurons. **(F-H)** Quantitative analyses of peak  
73 locomotion speed during stimuli (F), average locomotion speed after stimuli (G), and  
74 average locomotion speed before stimuli (H) of mice without (Ctrl) and with (TeNT)  
75 synaptic inactivation of SNc-projecting SC neurons. Scale bars are labeled in the graphs  
76 (A). Numbers of mice are indicated in the graphs (D-H). Data in (D-H) are means  $\pm$  SEM.  
77 Statistic analyses (F-H) are performed using Student t-test (n.s.  $P > 0.1$ ). For the P values,  
78 see Table S4.

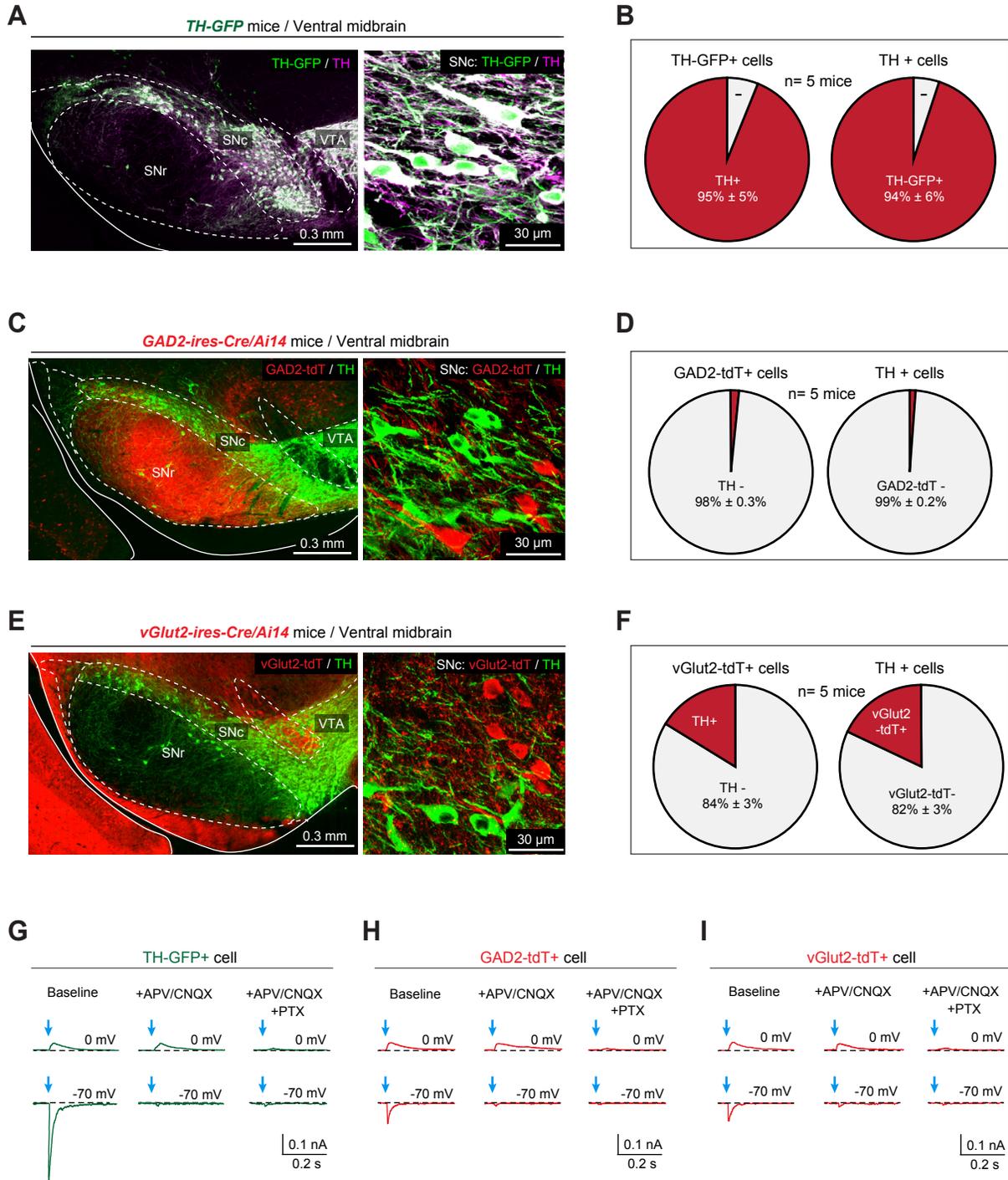


Figure S7 Huang et al., 2020

79 **Figure S7 Dopamine neurons are the primary postsynaptic target of the SC-SNc**  
80 **pathway.** (Related to Figure 5)

81 **(A, B)** Example micrographs (A) and quantitative analyses (B) showing that TH-GFP+  
82 cells and TH+ cells are largely overlapped in the SNc of *TH-GFP* mice. **(C, D)** Example  
83 micrographs (C) and quantitative analyses (D) showing that GAD2-tdT+ cells and TH+  
84 cells are largely segregated in the SNc of *GAD2-IRES-Cre/Ai14* mice. **(E, F)** Example  
85 micrographs (E) and quantitative analyses (F) showing that vGlut2-tdT+ cells and TH+  
86 cells are largely segregated in the SNc of *vGlut2-IRES-Cre/Ai14* mice. **(G-I)** Example  
87 traces of oIPSCs (*top*) and oEPSCs (*bottom*) from putative SNc dopamine neurons  
88 (TH-GFP+) (G), putative GAD2+ neurons (GAD2-tdT+) (H) and putative vGlut2+ neurons  
89 (vGlut2-tdT+) (I) with and without perfusion of antagonists of glutamate receptors  
90 (APV/CNQX) and GABA<sub>A</sub> receptor (PTX). Numbers of mice (B, D, F) are indicated in the  
91 graphs. Data in (B, D, F) are means  $\pm$  SEM. Scale bars are labeled in the graphs.

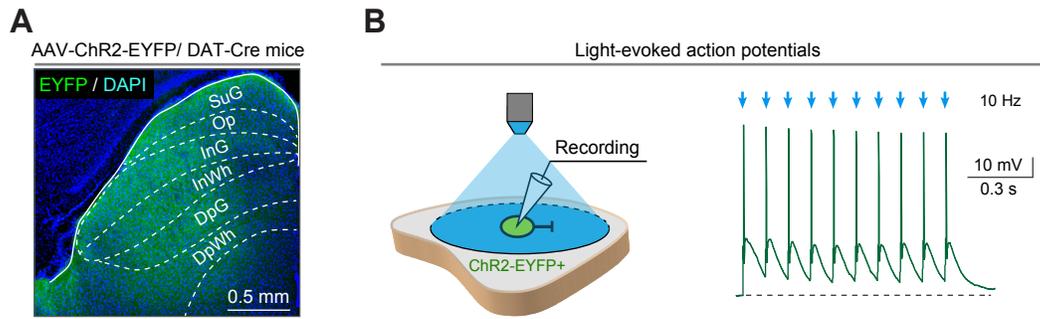


Figure S8 Huang et al., 2020

92 **Figure S8 SNc dopamine neurons mediate the effect of SC-SNc pathway activation.**

93 (Related to Figure 7)

94 **(A)** Example coronal section showing ChR2-EYFP expression in the SC. **(B)** Schematic  
95 diagram (*left*) and example trace (*right*) showing light pulses (2 ms, 473 nm, 10 Hz, 10  
96 pulses) reliably triggered action potential firing from ChR2-EYFP+ SC neurons in acute  
97 SC slices. Scale bars are labeled in the graphs.

98 **Movie S1 Action potential firing of SNc-projecting SC neurons during locomotion**

99 This movie shows that the action potential firing of a putative SNc-projecting SC  
100 neuron recorded with an optrode is correlated with locomotion. The action potentials  
101 have been sorted and their waveforms are displayed in the corner of the movie.

102 **Movie S2 Predatory hunting of an example control mouse without**  
103 **photostimulation of the SC-SNc pathway**

104 This movie shows behavioral analyses of predatory hunting of an example control  
105 mouse without photostimulation of the SC-SNc pathway. The left part of the screen  
106 displays the video taken by the overhead camera in parallel with computer-aided  
107 analyses of azimuth angle and PPD in real-time. The right part of the screen displays the  
108 time courses of azimuth angle, locomotion speed and PPD during predatory hunting in  
109 real-time. The approach episodes were labeled with shaded areas in orange.

110 **Movie S3 Predatory hunting of an example test mouse with photostimulation of**  
111 **the SC-SNc pathway**

112 This movie shows behavioral analyses of predatory hunting of an example test  
113 mouse with photostimulation of the SC-SNc pathway. The left part of the screen displays  
114 the video taken by the overhead camera in parallel with computer-aided analyses of  
115 azimuth angle and PPD in real-time. The right part of the screen displays the time  
116 courses of azimuth angle, PPD and locomotion speed during predatory hunting in  
117 real-time. The approach episodes were labeled with shaded areas in orange.

118 **Movie S4 Predatory hunting of an example control mouse without synaptic**  
119 **inactivation of SNc-projecting SC neurons**

120 This movie shows behavioral analyses of predatory hunting of an example control  
121 mouse without synaptic inactivation of SNc-projecting SC neurons. The left part of the  
122 screen displays the video taken by the overhead camera in parallel with computer-aided

123 analyses of azimuth angle and PPD in real-time. The right part of the screen displays the  
124 time courses of azimuth angle, locomotion speed and PPD during predatory hunting in  
125 real-time. The approach episodes were labeled with shaded areas in orange.

126 **Movie S5 Predatory hunting of an example test mouse with synaptic inactivation**  
127 **of SNc-projecting SC neurons**

128 This movie shows behavioral analyses of predatory hunting of an example test  
129 mouse with synaptic inactivation of SNc-projecting SC neurons. The left part of the  
130 screen displays the video taken by the overhead camera in parallel with computer-aided  
131 analyses of azimuth angle and PPD in real-time. The right part of the screen displays the  
132 time courses of azimuth angle, locomotion speed and PPD during predatory hunting in  
133 real-time. The approach episodes were labeled with shaded areas in orange.

134 **Movie S6 Defensive locomotion of an example control mouse without synaptic**  
135 **inactivation of SNc-projecting SC neurons**

136 This movie shows the overhead looming visual stimuli triggered escape followed by  
137 long-lasting freezing in an example control mouse without synaptic inactivation of  
138 SNc-projecting SC neurons.

139 **Movie S7 Defensive locomotion of an example test mouse with synaptic**  
140 **inactivation of SNc-projecting SC neurons**

141 This movie shows the overhead looming visual stimuli evoked immediate escape  
142 followed by long-lasting freezing in an example test mouse with synaptic inactivation of  
143 SNc-projecting SC neurons.

- 144 **Table S1 Mouse lines and reagents**
- 145 **Table S2 Summary of all experimental designs**
- 146 **Table S3 Summary of cell counting strategy**
- 147 **Table S4 Summary of statistical analyses**

**Table S1 Mouse lines and reagents**

| <b>Mouse Lines</b>               |                             |                             |
|----------------------------------|-----------------------------|-----------------------------|
| <i>vGlut2-IRES-Cre</i>           | JAX Mice                    | Stock No: 016963            |
| <i>GAD2-IRES-Cre</i>             | JAX Mice                    | Stock No: 010802            |
| <i>DAT-IRES-Cre</i>              | JAX Mice                    | Stock No: 006660            |
| <i>Ai14</i>                      | JAX Mice                    | Stock No: 007914            |
| <i>TH-GFP</i>                    | Hideyuki Okano Lab          | N/A                         |
| <b>Antibodies</b>                |                             |                             |
| Anti-EGFP                        | Abcam                       | ab290 / ab13970             |
| Anti-mCherry                     | Abcam                       | ab167453 / ab205402         |
| Anti-Glutamate                   | Sigma                       | G6642                       |
| Anti-GABA                        | Sigma                       | A2052                       |
| Anti-TH                          | Chemicon                    | AB152                       |
| <b>Chemicals and tracers</b>     |                             |                             |
| D-AP5 / CNQX / Picrotoxin        | Tocris                      | Cat. No. 0106 / 0190 / 1128 |
| TTX                              | Tocris                      | Cat. No. 1078               |
| 4-AP                             | Tocris                      | Cat. No. 0940               |
| CTB-555 / 488                    | Thermofisher Scientific     | Cat. No. C22843 / 22841     |
| Clozapine N-oxide (CNO)          | Enzo life sciences          | Cat. No. BML-NS105-0025     |
| Haloperidol                      | Sigma                       | Cat. No. H1512              |
| <b>AAV vectors</b>               |                             |                             |
| AAV-DJ-hSyn-GRAB-DA              | Shanghai Taitool Bioscience | N/A                         |
| AAV-DJ-hSyn-C1V1-mCherry         | Shanghai Taitool Bioscience | N/A                         |
| AAV-DJ-hSyn-DIO-EGFP-2A-TeNT     | Shanghai Taitool Bioscience | N/A                         |
| AAV-DJ-hSyn-DIO-hM4D-mCherry     | Brain VTA Inc.              | N/A                         |
| AAV-DJ-hSyn-ChR2-mCherry         | Brain VTA Inc.              | N/A                         |
| AAV-DJ-hSyn-mCherry              | Brain VTA Inc.              | N/A                         |
| AAV-DJ-hSyn-ChR2-EYFP            | Brain VTA Inc.              | N/A                         |
| AAV-DJ-hSyn-DIO-EGFP-Syb2        | Brain VTA Inc.              | N/A                         |
| AAV-DJ-hSyn-EGFP                 | Brain VTA Inc.              | N/A                         |
| AAV-DJ-hSyn-DIO-EGFP             | Brain VTA Inc.              | N/A                         |
| AAV2-retro-hSyn-mCherry-ires-Cre | Brain VTA Inc.              | N/A                         |

**Table S2 Summary of all experimental designs**

| Figures                      | Aims   | Mouse lines  | AAV injection & optical fiber implantation   | Type of data                                      |
|------------------------------|--|--|--|---|
| <b>Figure 1</b><br>Figure S1 | Cell-type-specific mapping of SC-SNc pathway   | <i>vGlut2-ires-Cre</i><br><i>GAD2-ires-Cre</i><br><br><i>WT</i>                | <i>AAV-hSyn-DIO-EGFP-Syb2</i> injected into the SC<br><br><i>CTB-555</i> injected into the SNc of WT mice  | Histology   |
| Figure S2                    | Segregation of SC-SNc and SC-VTA pathways  | <i>WT</i>  | <i>CTB-555 / CTB488</i> injected into the SNc and VTA, respectively<br><i>CTB-555 / CTB488</i> mixture injected into the SNc   | Histology   |
| Figure S3                    | Segregation of SC-SNc and SC-ZI pathways   | <i>WT</i>  | <i>CTB-555 / CTB488</i> injected into the SNc and ZI, respectively   | Histology   |
| <b>Figure 2</b><br>Figure S4 | SNc-projecting SC neurons encode locomotion speed  | <i>WT</i>  | <i>AAV-hSyn-ChR2-mCherry</i> injected into the SC<br><br>Optical fibers implanted above the SNc  | Single-unit recording                             |
| <b>Figure 3</b><br>Figure S5 | Activation of the SC-SNc pathway promotes appetitive locomotion  | <i>WT</i>  | <i>AAV-hSyn-ChR2-mCherry</i> injected into the SC<br><br>Optical fibers implanted above the SNc  | Behavior<br>Histology<br>Slice physiology         |
| <b>Figure 4</b><br>Figure S6 | The SC-SNc pathway is selectively required for appetitive locomotion   | <i>WT</i>  | <i>AAV2-retro-mCherry-IRES-Cre</i> injected into the SNc<br><i>AAV-hSyn-DIO-EGFP-2A-TeNT</i> injected into the SC<br><i>AAV-hSyn-DIO-EGFP</i> (Ctrl) injected into the SC                                      | Behavior<br>Histology                             |
| <b>Figure 5</b><br>Figure S7 | Genetic labeling of different cell types in the SNc<br>Synaptic innervation on SNc dopamine and non-dopamine neurons | <i>vGlut2-IRES-Cre</i><br><i>GAD2-IRES-Cre</i><br><i>Ai14</i><br><i>TH-GFP</i> | <i>AAV-hSyn-ChR2-mCherry</i> injected into the SC  | Histology<br>Slice physiology                     |
| <b>Figure 6</b>              | Activation of the SC-SNc pathway trigger dopamine release in the dorsal striatum                                     | <i>WT</i>  | <i>AAV-hSyn-C1V1-mCherry</i> injected into the SC<br><i>AAV-hSyn-GRAB-DA</i> injected into the dorsal striatum<br>Optical fibers implanted above the SNc and dorsal striatum                                   | Fiber photometry<br>Histology<br>Slice physiology |
| <b>Figure 7</b><br>Figure S8 | SNc dopamine neurons mediates the effect of the SC-SNc pathway activation  | <i>DAT-IRES-Cre</i>  | <i>AAV-hSyn-ChR2-EYFP</i> injected into the SC<br><i>AAV-hSyn-DIO-hM4D-mCherry</i> injected into the SNc<br><i>AAV-hSyn-DIO-mCherry</i> (Ctrl) injected into the SNc<br>Optical fibers implanted above the SNc | Behavior<br>Histology<br>Slice physiology         |

**Table S3 Summary of Cell Counting Strategy**

|                  |  |  |
|------------------|--|--|
| Brain region     | Superior Colliculus  | SNc & VTA  |
| Section type     | Coronal section (40 $\mu$ m)   | Coronal section (40 $\mu$ m)   |
| Section Range    | Bregma (-3.28 to -4.48)  | Bregma (-2.80 to -3.80)  |
| Total collection | Approximately 30 sections  | Approximately 25 sections  |
| Sampling         | To sample 1 section every 5 sections<br>to get 6 sections evenly spaced by 200 $\mu$ m | To sample 1 section every 5 sections<br>to get 5 sections evenly spaced by 200 $\mu$ m |

Table S4-1 Summary of statistical analyses

| Figure | Sample size (n)   | Statistical test | P values   |
|--------|---|------------------|--|
| 1E     | vGlut2-IRES-Cre = 5 mice<br>GAD2-IRES-Cre = 5 mice                        | One-Way ANOVA    | vGlut2-IRES-Cre vs. GAD2-IRES-Cre: P= 5.79939E-6 ***   |
| 3E     | Laser OFF<br>Ctrl = 5 mice<br>ChR2 = 5 mice                               | Student t-test   | ChR2 vs. Ctrl: P= 0.92794 n.s.   |
|        | Laser ON<br>Ctrl = 5 mice<br>ChR2 = 5 mice                                | Student t-test   | ChR2 vs. Ctrl: P= 1.1324E-7 ***  |
|        | Laser OFF<br>Ctrl = 5 mice<br>ChR2 = 5 mice                               | Student t-test   | ChR2 vs. Ctrl: P= 9.12091E-4 ***   |
| 3I     | OFF = 12 mice<br>ON = 12 mice   | Student t-test   | ON vs. OFF: P= 6.82E-09 ***  |
| 3J     | OFF = 12 mice<br>ON = 12 mice   | Student t-test   | ON vs. OFF: P= 7.61E-06 ***  |
| 3K     | OFF = 12 mice<br>ON = 12 mice   | Student t-test   | ON vs. OFF: P= 3.05E-06 ***  |
| 3L     | OFF = 12 mice<br>ON = 12 mice   | Student t-test   | ON vs. OFF: P= 0.15109 n.s.  |
| 3M     | OFF = 12 mice<br>ON = 12 mice   | Student t-test   | ON vs. OFF: P= 0.05989 n.s.  |
| 4E     | Ctrl = 12 mice<br>TeNT = 12 mice  | Student t-test   | TeNT vs. Ctrl: P= 0.0318 *   |
| 4F     | Ctrl = 12 mice<br>TeNT = 12 mice  | Student t-test   | TeNT vs. Ctrl: P= 0.41367 n.s.   |
| 4G     | Ctrl = 12 mice<br>TeNT = 12 mice  | Student t-test   | TeNT vs. Ctrl: P= 0.66311 n.s.   |
| 4H     | Ctrl = 12 mice<br>TeNT = 12 mice  | Student t-test   | TeNT vs. Ctrl: P= 0.00107 **   |
| 4I     | Ctrl = 12 mice<br>TeNT = 12 mice  | Student t-test   | TeNT vs. Ctrl: P= 0.00105 **   |
| 5E     | TH-GFP+ cells = 10 cells<br>GAD2-tdT+ cells = 10 cells                    | Student t-test   | TH-GFP+ oEPSC vs. oIPSC: P= 7.93268E-7 ***   |
|        |   |                  | GAD2-tdT+ oEPSC vs. EGFP+ oIPSC: P= 5.01684E-4 ***<br>oEPSC TH-GFP+ vs. GAD2-tdT+: P= 1.22279E-5 *** |
| 5G     | TH-GFP+ cells = 11 cells<br>GAD2-tdT+ cells = 10 cells                    | Student t-test   | TH-GFP+ oEPSC vs. oIPSC: P= 2.13383E-7 ***   |
|        |   |                  | GAD2-tdT+ oEPSC vs. oIPSC: P= 3.09254E-6 ***<br>oEPSC TH-GFP+ vs. GAD2-tdT+: P= 1.11192E-5 ***       |
| 6H     | GRAB-DA = 6 mice<br>EGFP = 6 mice   | One-Way ANOVA    | GRAB-DA vs. EGFP: P= 6.152478E-18 ***  |
| 6J     | Saline = 6 mice<br>Haldol 0.4 mg/kg = 6 mice<br>Haldol 1.6 mg/kg = 6 mice | One-Way ANOVA    | Saline vs. Haldol 0.4 mg/kg: P= 4.22687E-7 ***   |
|        |   |                  | Saline vs. Haldol 1.6 mg/kg: P= 7.35846E-12 ***  |

Table S4-2 Summary of statistical analyses

| Figure | Sample size (n)                             | Statistical test | P values                                  |
|--------|---|------------------|---|
| 7D     | Before CNO = 7 cells<br>After CNO = 7 cells | Student t-test   | After CNO vs. Before CNO: P= 2.41E-05 *** |
| 7E     | Saline-OFF = 8 mice<br>Saline-ON = 8 mice   | Student t-test   | Saline - OFF vs. ON: P= 8.63528E-6 ***    |
|        | CNO-OFF = 8 mice<br>CNO-ON = 8 mice         | Student t-test   | CNO - OFF vs. ON: P= 4.79306E-4 ***       |
| 7F     | Saline = 8 mice<br>CNO = 8 mice             | Student t-test   | CNO vs. Saline: P= 5.90024E-07 ****       |
| 7G     | Saline-OFF = 8 mice<br>Saline-ON = 8 mice   | Student t-test   | Saline - OFF vs. ON: P= 1.18523E-5 ***    |
|        | CNO-OFF = 8 mice<br>CNO-ON = 8 mice         | Student t-test   | CNO - OFF vs. ON: P= 0.01231 *            |
| 7H     | Saline = 8 mice<br>CNO = 8 mice             | Student t-test   | CNO vs. Saline: P= 7.82681E-6 ***         |
| S5C    | 2 Hz = 5 mice                               | Student t-test   | 5 Hz vs. 2 Hz: P= 1.25213E-4 ***          |
|        | 5 Hz = 5 mice                               | Student t-test   | 10 Hz vs. 5 Hz: P= 0.0204 *               |
|        | 10 Hz = 5 mice                              |                  |   |
| S6F    | Ctrl = 12 mice<br>TeNT = 12 mice            | Student t-test   | TeNT vs. Ctrl: P= 0.41367 n.s.            |
| S6G    | Ctrl = 12 mice<br>TeNT = 12 mice            | Student t-test   | TeNT vs. Ctrl: P= 0.66311 n.s.            |
| S6H    | Ctrl = 12 mice<br>TeNT = 12 mice            | Student t-test   | TeNT vs. Ctrl: P= 0.79514 n.s.            |